PATHOGENIC AND GENOMIC CHARACTERIZATION OF STRAINS OF *XANTHOMONAS CAMPESTRIS* CAUSING DISEASES OF CITRUS

By

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To my wife
Denise

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PATHOGENIC AND GENOMIC CHARACTERIZATION OF STRAINS OF XANTHOMONAS CAMPESTRIS CAUSING DISEASES OF CITRUS

By

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Strains of Xanthomonas campestris which cause diseases of citrus were characterized for their pathogenicity and their genomic relatedness. Strains of X. campestris pv. citrumelo, which cause Citrus bacterial spot, vary widely in their lesion expansion capabilities and their bacterial population development in and on citrus leaves. A host-strain interaction was observed among strains on Swingle citrumelo and Duncan grapefruit.

Restriction endonuclease patterns of infrequently occurring recognition sites in genomic DNA fragments separated by pulsed field gel electrophoresis were diverse for strains of X. campestris pv. citrumelo, whereas for strains of X. campestris pv. citri group A which causes Asiatic citrus canker and X. campestris pv. citri group B which causes Cancrosis B the restriction patterns were

relatively homogeneous. Strains of X. campestris pv. citrumelo, X. campestris pv. citri group A, and X. campestris pv. citri group B were all about 60% related to one another as determined by DNA reassociation. Strains of X. campestris pv. citrumelo averaged 88% similar to each other. Pathovars from hosts other than citrus, but known to cause lesions on citrus, were similar to strains of X. campestris pv. citrumelo by DNA reassociation. Pathovars from hosts other than citrus which do not cause lesions on citrus were not as related to X. campestris pv. citrumelo. Similarity values generated by DNA reassociation and Restriction Fragment Length Polymorphism (RFLP) analyses were not necessarily equivalent in comparisons of DNA reassociation values obtained here with RFLP analyses of other investigators.

All strains of X. campestris pv. citrumelo and X. campestris pv. citri group A examined possessed an hrp gene cluster which is interpreted to mean that even weakly aggressive strains of X. campestris pv. citrumelo are true pathogens. Strains of X. campestris pv. citrumelo were relatively similar in restriction patterns revealed by hybridization of an hrp probe as well as in carbohydrate utilization patterns. Comparisons of characterization methods cited here are illustrative of the advantages of using several techniques in the characterization of bacterial strains.

CHAPTER 1
INTRODUCTION

The possible repercussions of the introduction of Citrus canker have been understood since the disease was eradicated from Florida in 1933 (Stall and Seymour, 1983). Scientists, growers and regulators were aware of the losses Asiatic citrus canker could cause. Asiatic citrus canker can cause abscission of fruit and leaves and the lesions result in unmarketable fruit. The introduction of citrus canker would inevitably lead to an expensive eradication effort and shipment of fruit would be restricted.

Most citrus growing regions of the world are affected by one or more canker diseases of citrus (Koizumi, 1985). Four of these diseases are caused by bacteria classified as Xanthomonas campestris pv. citri (Civerolo, 1984). The diseases caused by X. campestris pv. citri have similar symptoms on citrus and citrus relatives, but differ in host range and geographical locations. The similarities among these diseases have resulted in the name citrus canker as an umbrella term for all diseases caused by X. campestris pv. citri. These diseases are Asiatic citrus canker, Cancrosis B, Mexican lime cancrosis and Citrus bacteriosis. For the purposes of this work, these diseases will all be referred

to as citrus canker diseases and the causal bacteria will be known as X. campestris pv. citri group A, group B, group C and group D, respectively.

In September 1984, a new disease of citrus, Citrus bacterial spot (Graham and Gottwald, 1988), was found in a central Florida citrus nursery (Schoulties et al., 1987). Research led to a basic understanding of this disease, but questions remained as to the ecology and epidemiology of its causal bacteria. Information regarding the population development of the causal agent was needed particularly to understand the potential for spread of the pathogen in the field. Little was known concerning the origin of this group of pathogens or the relationship of these strains to related pathogens in Florida. The genetic relatedness among strains of the causal organism and between these bacteria and strains of X. campestris pv. citri remains unclear.

The classification of the causal agent of Citrus bacterial spot has been the subject of controversy. The symptomatology of Citrus bacterial spot is similar to Citrus canker, but it can be differentiated from Citrus canker by the type of lesion formed. Therefore, it has been proposed that the causal bacteria be named X. campestris pv. citrumelo (Gabriel et al., 1989). Clear differences in symptomatology are justification for pathovar status (Dye et al. 1980) and therefore X. campestris pv. citrumelo terminology will be used here for the pathogen of Citrus

bacterial spot although it is recognized that a formal description of this pathovar has not been published. The elevation of the causal agent of Citrus bacterial spot to pathovar status raises questions regarding the classification of the groups of strains that cause Citrus canker. Justification for placing at least some of the groups of X. campestris pv. citri into separate pathovars may exist as was suggested by Gabriel et al. (1989), but more research needs to be done to define similarities and differences within these strains. Any study of the pathogen causing Citrus bacterial spot should, if possible, also include pathogens causing Citrus canker for comparison.

The primary objective of this work is to characterize bacterial strains responsible for Citrus canker and Citrus bacterial spot. Experiments were undertaken to assess the aggressiveness of strains of X. campestris pv. citri group A and X. campestris pv. citrumelo in growth chamber and greenhouse. Lesion expansion and development of external and internal populations of bacteria were evaluated on Duncan grapefruit and Swingle citrumelo to study host-strain interactions and relate leaf population dynamics to the ability of these strains to survive and spread in citrus nurseries. The genomic variation within strains of X. campestris pv. citrumelo and X. campestris pv. citri groups A and B and the differences between these groups is addressed. Techniques used to determine genomic relatedness

among strains include DNA reassociation, restriction endonuclease analysis, plasmid profiles, and hybridization analysis. These strains are also compared for differences in carbohydrate utilization patterns.

CHAPTER 2
REVIEW OF LITERATURE

Citrus canker diseases are an important factor in citrus production worldwide. Symptoms have been observed on all above ground parts of the citrus tree (Fawcett, 1936) with young tissue most susceptible (Stall et al., 1981; Stall et al., 1982). Citrus canker diseases vary in host range, symptomatology and geographical distribution (Civerolo, 1984). The causal organisms involved are all pathovars of Xanthomonas campestris (Civerolo, 1984). A similar disease, Citrus bacterial spot caused by X. campestris pv. citrumelo, affects nursery trees in Florida (Graham and Gottwald, 1988; Schoulties et al., 1987).

Disease Comparisons

The most important Citrus canker disease is Asiatic citrus canker, also known as Canker A, which probably originated in Asia (Fawcett, 1936). Today, however, Asiatic citrus canker is known in most areas where citrus is grown (Koizumi, 1985). The causal organism was originally described as Pseudomonas citri by Hasse (1915). The organism is now named X. campestris pv. citri, although recently a proposal to elevate pv. citri to species status has been published (Gabriel et al., 1989). Infection by X.

campestris pv. citri results in erumpent lesions on leaves, stems and fruits (Whiteside et al., 1988). Upon invasion of susceptible citrus tissue by strains of X. campestris pv. citri group A, swelling and degeneration of host cell walls occur, finally resulting in dissolution of plasmalemmas (Koizumi, 1979; Koizumi, 1988). The erumpent nature of Asiatic citrus canker lesions results from hypertrophy of parenchyma cells at the center of each lesion (Koizumi, 1976; Koizumi, 1977). After cell hypertrophy, bacterial multiplication increases and bacteria begin to ooze from lesions (Koizumi, 1976). Severe outbreaks of Asiatic citrus canker can lead to abscission of both fruit and leaves (Stall and Seymour, 1983). Abscission is probably due to ethylene production by X. campestris pv. citri multiplying within leaf tissue (Goto et al., 1980b). Asiatic citrus canker affects all commercial varieties although to varying degrees (Leite, 1990; Stall and Seymour, 1983).

Cancrosis B, first described as false canker, causes symptoms similar to Asiatic citrus canker, but is limited to South America where it was first observed in Argentina in 1928 (Ducharme, 1951). Cancrosis B affects primarily C. limon (lemon) in the field, although a number of citrus hosts can be affected if in close proximity to infected lemons (DuCharme, 1951). In side-by-side inoculations on grapefruit leaves, a strain of X. campestris pv. citri group B caused fewer lesions per cm^2 than a strain of X.

campestris pv. citri group A (Stall et al., 1981). Xanthomonas campestris pv. citri group B, varies physiologically from strains of X. campestris pv. citri group A and has been referred to as X. citri f. atypica (Rossetti, 1977). Due to a limited host range, X. campestris pv. citri group B is not considered as important a pathogen as X. campestris pv. citri group A (Stall et al., 1981).

In 1963, a citrus canker-like disease was observed in the state of São Paulo, Brazil (Rossetti, 1977). Although originally feared to be Asiatic citrus canker, the disease affected only Mexican lime. Mexican lime cancrosis, sometimes called Canker C, has only been observed in the state of São Paulo, Brazil, on Mexican lime. Symptoms on Mexican lime are similar to both Asiatic citrus canker and Cancrosis B. On grapefruit, hypersensitive-like symptoms were observed upon artificial inoculations with a Mexican lime cancrosis strain (Stall et al., 1981). The bacterium was originally classified as X. citri f. sp. aurantifolia (Namekata and Oliveira, 1971) but is now referred to as group C of X. campestris pv. citri (Civerolo, 1984). It is of limited occurrence and thus is not considered of economic importance.

A disease known as citrus bacteriosis or leaf-and twig-spot disease was first observed on Mexican lime trees in Mexico in 1981 (Rodriguez G. et al., 1985). Symptoms were similar to Asiatic citrus canker, but no lesions have been

observed on fruit. Citrus bacteriosis appears to be concentrated in the Pacific coastal regions of Mexico (Rodriguez G. et al., 1985). Strains of X. campestris have been inconsistently isolated from lesions (Rodriguez G. et al., 1985) and based on pathogenicity were classified as X. campestris pv. citri. The disease is most prevalent during the dry season, unlike most citrus canker diseases (Stapleton and Garza-Lopez, 1988). It has been suggested that Alternaria sp. may be the causal agent of this disease (Stapleton and Garza-Lopez, 1988). Recently, it has been proposed that strains of X. campestris pv. citri groups B, C and D be placed in X. campestris pv. aurantifolia (Gabriel et al., 1989).

Canker-like symptoms were discovered on trees in a Florida citrus nursery in September, 1984 (Schoulties et al., 1987). Lesions occurred on leaves and stems and in artificial inoculations, fruit were susceptible (Graham et al., 1990b) and the host range seemed to be similar to that of Asiatic citrus canker (Schoulties et al., 1987). Symptoms differed from citrus canker diseases in that lesions on leaves and fruit were flat with very prominent watersoaked margins (Schoulties et al., 1987). Hypertrophic tissue has not been associated with these lesion types (Dienelt and Lawson, 1989; Lawson et al., 1989). These symptom differences have led to the proposed name Citrus bacterial spot for the disease (Graham and Gottwald, 1988).

Genetic differences, based on RFLP analysis, from X. campestris pv. citri resulted in the proposal of the name X. campestris pv. citrumelo for the causal organism (Gabriel et al., 1989). Citrus bacterial spot has been restricted to Florida citrus nurseries occurring mainly on Swingle citrumelo and grapefruit varieties (Graham and Gottwald, 1990). Citrus cultivars derived from hybrids with Poncirus trifoliata are susceptible to Citrus bacterial spot in artificial inoculations (Graham et al., 1990a).

There exist two additional xanthomonad strains that have been reported to cause disease on rutaceous hosts. Xanthomonas campestris pv. bilvae causes disease on the following hosts: Aegle marmelos (L.) Correa; C. aurantifolia (Christm.) Swingle, Feronia elephantum and Limonia acidissima L. (Dye and Lelliote, 1974; Leyns et al., 1984). The name X. bilvae was not included on the approved lists of pathovar names (Dye et al., 1980) and is not listed by Bradbury (1984). Also not included by either Bradbury (1984) or Dye and Lelliote (1974), is X. campestris pv. feronia which exists in the American Type Culture Collection. This is a pathogen of Feronia spp. but, there is no record of a list of hosts for this strain (E. L. Civerolo, personal communication). The relationship of these strains to any group of X. campestris pv. citri or X. campestris pv. citrumelo is not known.

In addition to symptomatology, host range, and geographical distribution, the causal agents of the Citrus

canker diseases and Citrus bacterial spot differ in a number of other ways. They differ in their epidemiology, phenotypic characteristics, serological determinants, genetic variation, isozyme variation and fatty acid profiles. Collectively, these methods indicate these pathogens are distinct (Civerolo, 1985b; Civerolo, 1988).

Epidemiological Comparisons

Citrus canker diseases are generally more frequent in climates where rainy periods and warm weather coincide (Peltier and Frederick, 1926). Wind-driven rain is critical to the spread of strains of X. campestris pv. citri group A (Gottwald et al., 1989; Stall et al., 1980) and allows stomatal entry of the causal bacteria by watersoaking leaves. Short distance spread of the bacteria may be due to wind and rain which cause the spread of leaf surface bacteria (Danós et al., 1984; Gottwald et al., 1988b), especially those bacteria exuded onto leaf surfaces after rain events (Stall et al., 1980). The spread of X. campestris pv. citri group A is often directional, most often according to the prevailing winds (Gottwald et al., 1989). Thus, wind breaks have been effectively used as a method of control (Civerolo, 1981; Leite, 1990).

In contrast to Asiatic citrus canker, X. campestris pv. citrumelo is most often spread mechanically (Gottwald and Graham, 1990). Weakly aggressive strains of X. campestris pv. citrumelo were spread mechanically down rows, while a

highly aggressive strain was apparently capable of wind driven spread (Gottwald and Graham, 1990; Gottwald and Graham, unpublished). Citrus bacterial spot appears not to be spread directionally in response to windblown rain in both simulated grove and nursery conditions (Gottwald et al., 1988a). However, a highly aggressive strain did spread in response to artificially generated wind (Gottwald and Graham, unpublished data). Outbreaks of Citrus bacterial spot have been primarily limited to citrus nurseries, in contrast with Asiatic citrus canker which also affects mature trees (Schoulties et al., 1987). As with citrus canker diseases, Citrus bacterial spot has been observed primarily during warm, rainy months (Graham and Gottwald, 1990).

Phenotypic Comparisons

Although all strains of X. campestris share the physiological characters of the species, other properties such as carbohydrate utilization and phage sensitivity vary among strains of X. campestris pv. citri and X. campestris pv. citrumelo. Strains of X. campestris pv. citri group B are difficult to grow on common bacteriological media, but a few colonies from such isolations appear that are capable of growth on nutrient agar (Canteros de Echenique et al., 1985; Stall et al., 1981). All other strains of X. campestris pv. citri and X. campestris pv. citrumelo grow well on nutrient agar. Strains of X. campestris pv. citri group B capable of

growth on nutrient agar were not able to utilize lactose or maltose in contrast with strains of X. campestris pv. citri group A (Goto et al., 1980a). Other investigators noted the differential carbon utilization of strains of X. campestris pv. citri group A and B (Alcaraz, 1977; Gotuzzo and Rossi, 1968) and proposed the name X. citri f. atypica for the latter strains (Gotuzzo and Rossi, 1968). Comparisons of strains of X. campestris pv. citri group A and X. campestris pv. citrumelo with physiological traits have been reported, but the tests did not differentiate between these pathovars (Gabriel et al., 1989). Susceptibility of X. campestris pv. citri group B to phage Cp3 separated those strains from strains of X. campestris group A (Goto et al., 1980a). Although most strains of X. campestris pv. citri group A were susceptible to phages Cp1 and Cp2, these phages did not attack strains of X. campestris pv. citri group B and C (Namekata, 1975).

Serological Comparisons

Strains of the different groups of X. campestris pv. citri have also been distinguished by serological techniques. Using indirect ELISA (Enzyme Linked Immunosorbent Assay) tests, strains of X. campestris pv. citri group A could be differentiated from strains of X. campestris pv. citri groups B and C, although the difference between strains of X. campestris pv. citri groups B and C was not conclusive (Civerolo and Helkie, 1981). Bach (1981)

distinguished strains of X. campestris pv. citri group A from strains of X. campestris pv. citri group C with ELISA. Strains of X. campestris pv. citri groups A and D may be related since five of six strains of X. campestris pv. citri group D reacted positively in ELISA assays using anitsera to X. campestris pv. citri group A (Rodriguez G. et al., 1987). Strains of X. campestris pv. citri group A and B were differentiated serologically with immunodiffusion and immunoelectrophoresis (Messina, 1980; Goto et al., 1980a). Strains of X. campestris pv. citri group C differed from strains of X. campestris pv. citri group A in agglutination, precipitation, gel-diffusion and immunoelectrophoresis tests (Namekata and Oliveira, 1971). Based on these results, as well as physiological and pathogenicity tests, Namekata and Oliveira (1971) suggested the name X. citri f. sp. aurantifolia as the causal agent of Mexican lime cancrosis.

Strains of X. campestris pv. citri groups A, B, and C were differentiated by monoclonal antibodies, and few other xanthomonads reacted to any of the monoclonal antibodies tested (Benedict et al., 1985). Monoclonal antibodies (from unstable hybridomas) specific for strains of X. campestris pv. citri group C have been obtained (Alvarez et al., 1987). These investigators found monoclonal antibodies that distinguished X. campestris pv. citri groups A and B. However, a monoclonal antibody that reacted with slow growing X. campestris pv. citri group B also reacted with X.

campestris pv. citri group C and M3 strain of X. campestris pv. citri group D from Mexico (Alvarez et al., 1987). Strains of X. campestris pv. citri group A shared a common epitope and were distinct from strains of X. campestris pv. citrumelo (Alvarez et al., 1990). Strains of the latter pathovar are diverse and sometimes share epitopes with several other pathovars of X. campestris (Alvarez, 1990). A monoclonal antibody made to strain X-4600 of X. campestris pv. citrumelo did not react with any groups of X. campestris pv. citri and only half of the strains of X. campestris pv. citrumelo tested (Permar and Gottwald, 1989). Thus, strains of X. campestris pv. citrumelo are serologically distinct from groups of X. campestris pv. citri (Alvarez et al., 1990; Permar and Gottwald et al., 1989).

Genetic Comparisons

Xanthomonas campestris pv. citrumelo apparently differs from X. campestris pv. citri, and the three groups of the latter pathovar can be separated on the basis of pathogenic, physiological and serological characteristics. All of these characteristics have a genetic basis, but the amount of genetic variation can not be ascertained by the above techniques. Several techniques have been used to examine genetic differences among these groups. At least seven distinct plasmids exist for X. campestris pv. citri which include separate profiles for strains of each group (Civerolo, 1985a). Some strains of X. campestris pv.

citrumelo were reported to have plasmids of 41 or 67 kilobase pairs (Gabriel et al., 1989). In the latter study, plasmid profiles were not compared with profiles of strains in groups of X. campestris pv. citri, but at least one plasmid size class (67 kilobase pairs) was found in strains of both X. campestris pv. citrumelo and strains of X. campestris pv. citri group A (Civerolo, 1985a; Gabriel et al., 1989).

Restriction endonuclease analysis of frequently occurring recognition sites qualitatively distinguished between groups of X. campestris pv. citri and X. campestris pv. citrumelo (Hartung and Civerolo, 1987). This technique also revealed the heterogeneity of strains of X. campestris pv. citrumelo and the uniformity of each group of X. campestris pv. citri.

Whereas the restriction endonuclease analysis cited above yielded qualitative differences, restriction fragment length polymorphism (RFLP) analysis allows quantitative comparisons to be made. Based on RFLP data, strains of each group of X. campestris pv. citri were relatively uniform, whereas strains of X. campestris pv. citrumelo were more diverse (Gabriel et al., 1988, 1989; Graham et al., 1990c; Hartung and Civerolo, 1989). Strains of X. campestris pv. citri group A and B were readily differentiated; however, the degree of similarity between these groups depended upon the individual study (Gabriel et al., 1988; Hartung and

Civerolo, 1989). Strains of X. campestris pv. citri groups B and D were similar in RFLP comparisons (Gabriel et al., 1989; Hartung and Civerolo, 1989). Gabriel et al. (1989) placed X. campestris pv. citri groups B, C, and D together as X. campestris pv. aurantifolia; however, Hartung and Civerolo (1989) found a significant difference between the strain of X. campestris pv. citri group C and the B and D groups. In all three investigations, the groups of X. campestris pv. citri were separated from strains of X. campestris pv. citrumelo by RFLP analysis (Gabriel et al., 1988, 1989; Hartung and Civerolo, 1989).

Strains of X. campestris isolated from citrus and noncitrus hosts, including strains of X. campestris pv. citrumelo, have been compared by RFLP analysis (Graham et al., 1990c). Those strains capable of eliciting a necrotic response on citrus, whether of citrus origin or not, were more related than strains which did not elicit a necrotic reaction. This study emphasized the diversity of X. campestris pv. citrumelo and the host range and genetic overlap between these strains and other pathovars.

Fatty Acid Comparisons

Graham et al. (1990c) also compared fatty acid profiles of strains of X. campestris of citrus and noncitrus origin. As with RFLPs, those strains capable of lesion formation on citrus were more similar to X. campestris pv. citrumelo than nonlesion-forming strains, regardless of whether they were isolated from citrus.

Isozyme Comparisons

The three groups of X. campestris pv. citri and strains of X. campestris pv. citrumelo were also investigated using isozyme analysis (Kubicek et al., 1989). The results of this study are in general agreement with the above discussion of genetic variation. Isozyme analysis revealed that strains of X. campestris pv. citri group A and B were relatively homogeneous, whereas strains of X. campestris pv. citrumelo were diverse. In addition, isozyme analysis clearly differentiated among the groups of X. campestris pv. citri and strains of X. campestris pv. citrumelo.

Although Asiatic citrus canker, Cancrosis B and Mexican lime cancrosis cause similar leaf, stem and fruit spots on citrus, the diseases and their causal agents differ in several characteristics. The current situation whereby three diseases of citrus exist within X. campestris pv. citri leads to confusion. Perhaps sufficient differences in host range and/or aggressiveness exist to justify placing the causal agents of these three diseases in separate pathovars. The demonstration of pathogenicity through artificial inoculation is often difficult; therefore, pathogenicity characters should be complemented with physiological tests, bacteriophage sensitivities or genetic determinants which correspond to pathogenicity characters.

CHAPTER 3
PATHOGENIC CHARACTERIZATION

In Florida, two diseases of citrus are caused by xanthomonads. Asiatic citrus canker, caused by Xanthomonas campestris pv. citri group A Hasse (Syn. Xanthomonas citri Hasse) strains, which has a worldwide distribution, causes erumpent lesions on leaves, stems, and fruits of many citrus cultivars (Civerolo, 1984). Economic losses from citrus canker may result from fruit lesions which decrease fresh fruit value, abscission of fruits or leaves, and regulatory measures (e.g., shipping restrictions, eradication) designed to halt the spread of the disease (Civerolo, 1984; Stall and Seymour, 1983). Citrus bacterial spot, caused by X. campestris pv. citrumelo (Gabriel et al., 1989) (syn. X. campestris pv. citri strain E), has only been associated with nursery plants in Florida (Schoulties et al., 1987). The majority of outbreaks have occurred on Swingle citrumelo (Poncirus trifoliata (L.)Raf. x Citrus paradisi Macf.) or grapefruit varieties (C. paradisi). Lesions on stems and leaves are flat, variously watersoaked and/or necrotic (Graham and Gottwald, 1990). Although X. campestris pv. citrumelo has not caused severe disease loss, regulatory actions to eradicate citrus canker have been applied to

citrus bacterial spot due to uncertainty surrounding the biological relationships of these two diseases and their causal bacteria (Schoulties et al., 1987).

The epidemiological significance of strains of X. campestris pv. citrumelo compared to strains of X. campestris pv. citri group A is not fully resolved. The strains causing citrus bacterial spot have been classified into aggressiveness types based on the extensiveness of watersoaking and necrosis on wound-inoculated leaves (Graham and Gottwald, 1990) and by different interactions on Swingle citrumelo, Duncan grapefruit and other citrus cultivars (Graham and Gottwald, 1990; Graham et al., 1990a). The most severe reactions are associated with the highly aggressive strain on Swingle citrumelo. Graham et al. (1990c) suggested, based on host reactions and the genetic uniformity of the highly aggressive strains, that they are the only strains that should be classified as X. campestris pv. citrumelo.

Less aggressive strains, however, have been found on several citrus cultivars in field nurseries (Graham and Gottwald, 1990). When leaves of Swingle citrumelo and Duncan grapefruit are inoculated, lesions are readily formed and undergo limited expansion (Graham and Gottwald, 1990; Graham et al., 1990a). While the highly aggressive strains appear to be spread by wind blown rain in nurseries, the less aggressive strains are spread only mechanically down

nursery rows on wounded plants (Gottwald and Graham, 1990; Graham and Gottwald, 1990). Although differences in the internal populations of different aggressiveness types on citrus cultivars have been identified in the greenhouse (Graham et al., 1990a), such differences have not been demonstrated in the field. In addition, it is not clear how external populations differ among aggressiveness types. Leaf surface populations are important in the spread and development of citrus canker (Danós et al., 1984; Gottwald, et al., 1988b; Stall et al., 1980).

The relationships were investigated among internal and external bacterial populations and lesion expansion on leaves for X. campestris pv. citri group A and X. campestris pv. citrumelo on Swingle citrumelo and grapefruit, the cultivars most commonly affected by citrus bacterial spot in Florida nurseries. Different inoculation and sampling methods were used in an attempt to relate aggressivess of the two pathovars and strains of X. campestris pv. citrumelo to differences in population dynamics in leaves and the availability of leaf surface populations for spread. A preliminary report of a portion of this study has been published (Egel et al., 1988).

Materials and Methods

Bacterial strains. All strains were isolated by the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI) except for X. campestris

pv. citri group A strain MF23P which was isolated by T. Riley. Three strains of X. campestris pv. citrumelo F1 (DPI no. 84-3048), F6 (DPI no. 84-3401), and F100 (DPI no. 85-12869) were previously determined to be highly, moderately, and weakly aggressive on Swingle citrumelo and Duncan grapefruit respectively (Graham and Gottwald, 1990). Based on morphological and physiological tests which identified strains to the species X. campestris (Schoulties et al., 1987) and their reaction on host to determine pathovar (Gabriel et al., 1989; Graham and Gottwald, 1990; Graham et al., 1990a), strains were classified as either X. campestris pv. citri group A or X. campestris pv. citrumelo. The two strains of X. campestris pv. citri group A, MF23P and 9771, were equally aggressive in wound inoculations like those previously reported (Graham and Gottwald, 1990). For inoculation, bacteria were cultured 12-15 hr in Difco (Detroit, MI) nutrient broth (NB), harvested by centrifugation at 8000 g for 15 min, and resuspended in sterile tap-water. Bacteria were adjusted turbidimetrically to 1 x 10^8 colony forming units (cfu)/ml (0.1 absorbance at 600 nm) and appropriate dilutions made. Final populations were determined by plating on Difco nutrient agar or nutrient-glucose agar (Difco nutrient agar amended with 0.1 g glucose/L).

Population dynamics after injection-infiltration of leaves. Experiments were conducted with strain 9771 of X.

campestris pv. citri group A and the 3 strains of X. campestris pv. citrumelo F1, F6 and F100 on Swingle citrumelo and Duncan grapefruit in a quarantine greenhouse at DPI, Gainesville, FL. Seedlings were cut back to produce uniformly susceptible immature leaves (3/4 to fully expanded). These were inoculated by injecting 10^4 cfu/ml into the mesophyll of the leaf with a 26 gauge needle which resulted in initial populations of 10^2 to 10^3 cfu/cm^2 of leaf area. Populations of bacteria per cm^2 were estimated at 0, 1, 5, 10, 20, 30 and 40 days in a leaf disk (0.6 or 0.28 cm^2) harvested from a randomly chosen site within the inoculated area and grinding the tissue in 1 ml phosphate buffer (0.075 M, pH 7.0) in a glass tissue homogenizer. Dilutions of the extracts were plated on nutrient agar amended with chlorothalonil (Bravo 720, a.i., 12 mg/L). Populations were expressed as the log transformation of cfu/cm^2 of leaf area. Each treatment was replicated five times and each replicate was represented by one leaf per seedling. Each experiment involving a particular host was repeated at least once.

Population dynamics under growth chamber conditions. Replicate experiments were conducted with Swingle citrumelo seedlings in a Percival Dew Chamber (Model I-35 DL, Percival, Boone IA) at USDA quarantine facilities in Plymouth, FL. Photoperiods were 10 hr light (28 C, 92% relative humidity) and 14 hr dark (30 C, 96% relative

humidity). Although dew formed daily, mist was applied for 4 hr prior to sampling thereby augmenting moisture on the leaf surface. The experimental design was a randomized complete block with five replications per treatment; seedlings were placed randomly on chamber shelves and rearranged every third day.

Six-month old seedlings were cut back to produce uniformly susceptible immature leaves which were inoculated by puncturing each side of the midvein with a 26 gauge syringe needle and applying a 10 μl drop of a 10^8 cfu/ml suspension to the adaxial side of the puncture wounds. All four strains (MF23P, F1, F6, and F100) were inoculated on to each leaf with a minimum of five leaves treated.

Internal populations in leaves were determined by removing lesions with a cork borer. Lesion diameter was measured to the nearest 0.5 mm with a micrometer. Tissue was ground in 2 ml of phosphate buffer, and the suspension plated on KCB semiselective medium (NA plus kasugamycin 16.0 mg/L, cephalexin 16.0 mg/L, and chlorothalonil (Bravo 720) 12.0 mg/L) (Graham and Gottwald, 1990). Lesions were sampled at 10, 20, 32, and 41 days after inoculation. Populations were expressed as the log cfu/lesion.

External populations on leaves were evaluated by absorbing the moisture from the adaxial surface of individual lesions with a sterile, cotton swab after an overnight dew cycle. Swabs were placed in 5 ml of phosphate

buffer, sonicated for 3 min, and incubated for 30 min on a rotary shaker at room temperature. Sonication did not adversely affect the viability of bacteria. The solution was plated onto KCB media. External bacterial populations were expressed as log cfu/lesion and sampling times were as above.

Population dynamics under simulated nursery conditions. Field experiments included X. campestris pv. citrumelo strains F1, F6, and F100 on Swingle citrumelo and Duncan grapefruit at a quarantine facility in Hastings, FL. The use of X. campestris pv. citri group A strains in field experiments was prohibited by federal and DPI quarantine regulations.

Simulated nurseries consisted of 4 rows of 25 seedlings (20-30 cm tall) of each cultivar spaced 10 cm apart within rows and 30 cm between rows with 10 m between plots. Each plot was separated by nylon screening as wind breaks to prevent spread of bacteria among plots. Plants were inoculated by mechanically rubbing a 10^8 cfu/ml mixture of each strain with carborundum onto the upper and lower surface of leaves. The experiment was a 3 x 2 factorial where each treatment consisted of a strain-cultivar combination. Daily minimum temperatures during the experiment (spring 1989) ranged from 8 C to 23 C and maximum temperatures ranged from 25 C to 35 C. Total rainfall for the experimental period was 117 mm. Seedlings were

inoculated on 1 September 1988 and 10 May 1989. Although conducted during different times of the year, the results of the two experiments reinforced each other and data from the second experiment are reported.

After symptoms appeared (in ca. 14 days), seven seedlings were chosen as replicates in each treatment with five lesions chosen on each seedling. These lesions were measured to the nearest 0.5 mm with a micrometer at approximately 7 day intervals until 56 days post-inoculation. To estimate external populations, lesions on the adaxial surface of leaves were swabbed at about 8-9 am when dew was present. The moisture present on all five lesions of a single plant was absorbed onto a single sterile cotton swab. On some dates dew formation was too low to supply a sample, in which case it was augmented by overhead irrigation for 30 min prior to sampling (on days 15 and 36). Each swab absorbed, on average, 25 μl moisture per seedling. Swabs were placed in 5 ml of phosphate buffer and held at 4 C (not more than 24 hr) until samples could be plated. Vials were vortexed 10-20 sec, 0.5 ml removed and plated at the appropriate dilutions on KCB medium.

Internal populations in leaf lesions were estimated from seven randomly chosen lesions from each treatment at 7 day intervals until 70 days post-inoculation. Lesions were removed with a cork borer and held at 4 C until processed. Lesions were measured to the nearest 0.5 mm with a

micrometer and then ground in 2 ml of phosphate buffer in a tissue homogenizer and the extract plated onto KCB media.

Statistical analysis. Population and lesion diameter data for each date were compared by ANOVA; if the F test was significant at the 0.05 level, means were compared by Tukey's HSD-procedure (α=0.05) for each date. Both GLM and Tukey procedures were run using SAS (Statistical Analysis Systems, Cary, NC). In the simulated nursery experiment, significant interactions were present at the α=0.05 level for cultivar x strain on several dates; therefore, strains were compared separately on each cultivar.

Results

Population dynamics after injection-infiltration of leaves. Strains F1 and F6 of X. campestris pv. citrumelo caused indistinguishable flat lesions with watersoaked, necrotic centers, and chlorotic halos 5-7 days after inoculation by injection-infiltration. Lesions elicited by X. campestris pv. citrumelo strain F100 developed slowly (8-10 days after inoculation) as small, reddish, raised spots and expanded into slightly raised necrotic areas with little watersoaking or chlorosis. In contrast, lesions caused by X. campestris pv. citri group A strain 9771 appeared as raised green spots (5-7 days after inoculation), expanded quickly and eventually became erumpent, necrotic lesions with marginal watersoaking and chlorosis.

On both hosts, internal leaf populations of strains 9771, F1, and F6 increased rapidly up to 5 days, peaked by 20 days, and slowly declined thereafter (Fig. 3-1). Except on day five for Swingle citrumelo, the populations of these three strains were not different on either cultivar. Nor were cultivar-strain interactions detected. Strain F100 reached populations that were ca. 2 log units lower than any other strain; this difference was generally significant for all dates and both cultivars (Fig. 3-1). In Duncan grapefruit, strain F100 was not detected at 30 and 40 days after inoculation (Fig. 3-1B).

Population dynamics under growth chamber conditions. More differences among strains in bacterial population dynamics in and on lesions were detected using the pin-prick inoculation method than were detected by the injection-infiltration method (Fig. 3-1, 3-2). Internal leaf populations of X. campestris pv. citri group A strain MF23P and X. campestris pv. citrumelo strain F1 in Swingle citrumelo were generally not different (Fig. 3-2A) but were significantly higher than populations of strain F6 by day 30. As previously indicated by injection-infiltration, strain F100 generally produced lower populations than any other strain (Fig. 3-2A).

There were fewer differences among strains in external populations on leaves sampled by absorbing dew off of the lesions with swabs. At 10 and 20 days, populations of X.

campestris pv. citri group A strain MF23P were higher than strains of X. campestris pv. citrumelo, but populations of strains F1 and F6 were similar (Fig. 3-2B). Moreover, internal populations of strain F100 significantly differed from strains F1 and F6 only on days 20 and 32 (Fig. 3-2B). Thus, under dew-forming conditions in the growth chamber, external populations among the strains were not as readily distinguishable as internal populations.

Expansion of the erumpent lesions elicited by X. campestris pv. citri group A strain MF23P was not comparable to the flat lesions produced by the strains of X. campestris pv. citrumelo (Fig. 3-2C). Strains F1 and F6 produced larger lesions than strain F100 and strain MF23P. Lesion expansion by strains F1 and F6 was indistinguishable as was that of strains F100 and MF23P.

Considering both X. campestris pv. citri group A and X. campestris pv. citrumelo strains, external populations were well correlated with internal populations (r=0.64) at 41 days but were less correlated with lesion diameter (r=0.14). If X. campestris pv. citri group A, which did not form a comparable lesion type, was excluded from the analysis, then internal and external populations and lesion diameters of strains of X. campestris pv. citrumelo were all well correlated with each other at 41 days (Table 3-1). Those correlations which included external populations tended to be lower.

Population dynamics under simulated nursery conditions. There were significant host-strain interactions among the three strains of X. campestris pv. citrumelo on the two cultivars when populations in lesions produced by wound inoculation were compared (Fig. 3-3A, 3-3B). Internal populations usually exceeded 10^6 cfu/lesion for strain F1 on Swingle citrumelo and were higher than populations of all other host-strain combinations. Internal populations in lesions produced by F1 and F6 on Duncan grapefruit generally did not differ significantly. Strain F100 populations were consistently below 10^4 cfu/lesion and were not detectable after day 49 and 56 on Duncan grapefruit and Swingle citrumelo, respectively (Fig. 3-3A, 3-3B). Overall, populations in lesions were stable for strain F1 on both hosts, but fluctuated for strain F6 and were constantly dropping for strain F100.

Bacterial populations sampled from the dew on the surface of leaf lesions followed the same general trends as internal populations (Fig. 3-3C and 3-3D). For Swingle citrumelo, external populations of strain F1 were usually significantly higher than populations of F6 and generally higher than any other host-strain combination (Fig. 3-3C and 3-3D). On Duncan grapefruit, populations of strains F1 and F6 were not significantly different throughout the sampling period (Fig. 3-3D). External populations on lesions produced by strain F100 were generally less than 10

Table 3-1. Correlation of internal and external populations and lesion diameters at 41 days for strains of *Xanthomonas campestris* pv. *citrumelo* of different aggressiveness types on Swingle citrumelo (SC) and Duncan grapefruit (DG) in growth chamber and field experiments.

	Growth chamber	Field	
Correlation of:	SC	SC	DG
Internal *vs* external	0.56	0.79	0.84
Internal *vs* lesion diameter	0.83	0.87	0.94
External *vs* lesion diameter	0.55	0.85	0.88

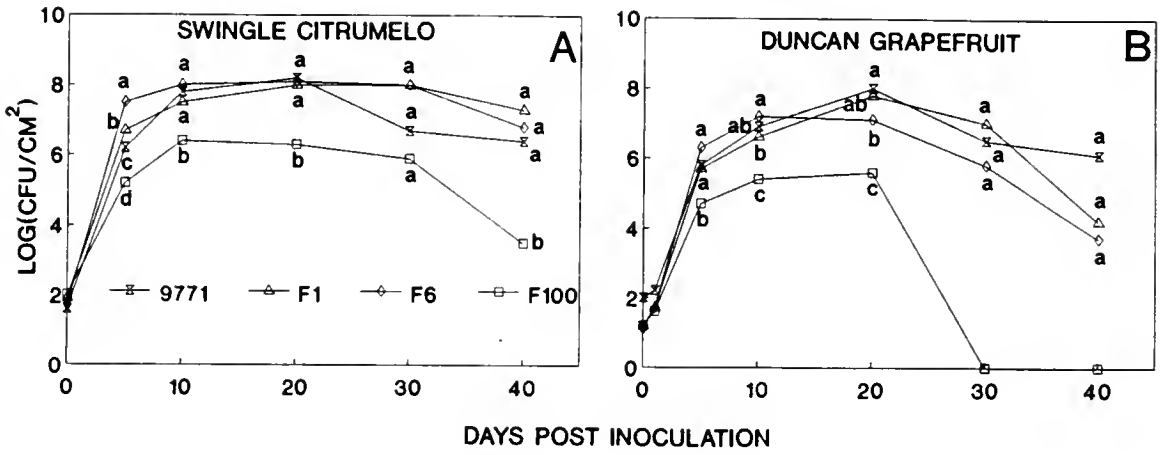


Fig. 3-1. Populations of Xanthomonas campestris pv. citri strain 9771 and X. c. citrumelo strains F1, F6, and F100 in leaves of greenhouse-grown seedlings of Swingle citrumelo (A) and Duncan grapefruit (B) leaves inoculated by an injection-infiltration method in the greenhouse. Each data point is the mean of five replications. Mean values on each sampling date are significantly different from means for treatments on that same date that are not accompanied by the same letter by Tukey's HSD, α=0.05.

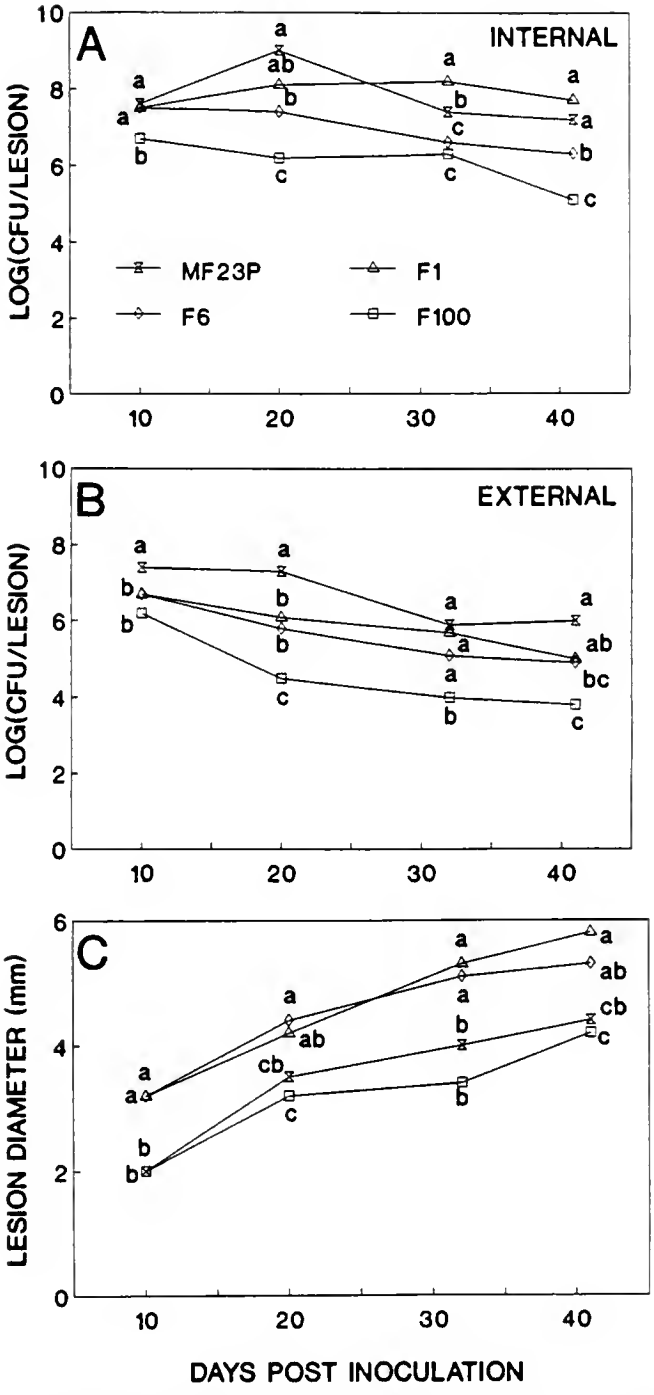
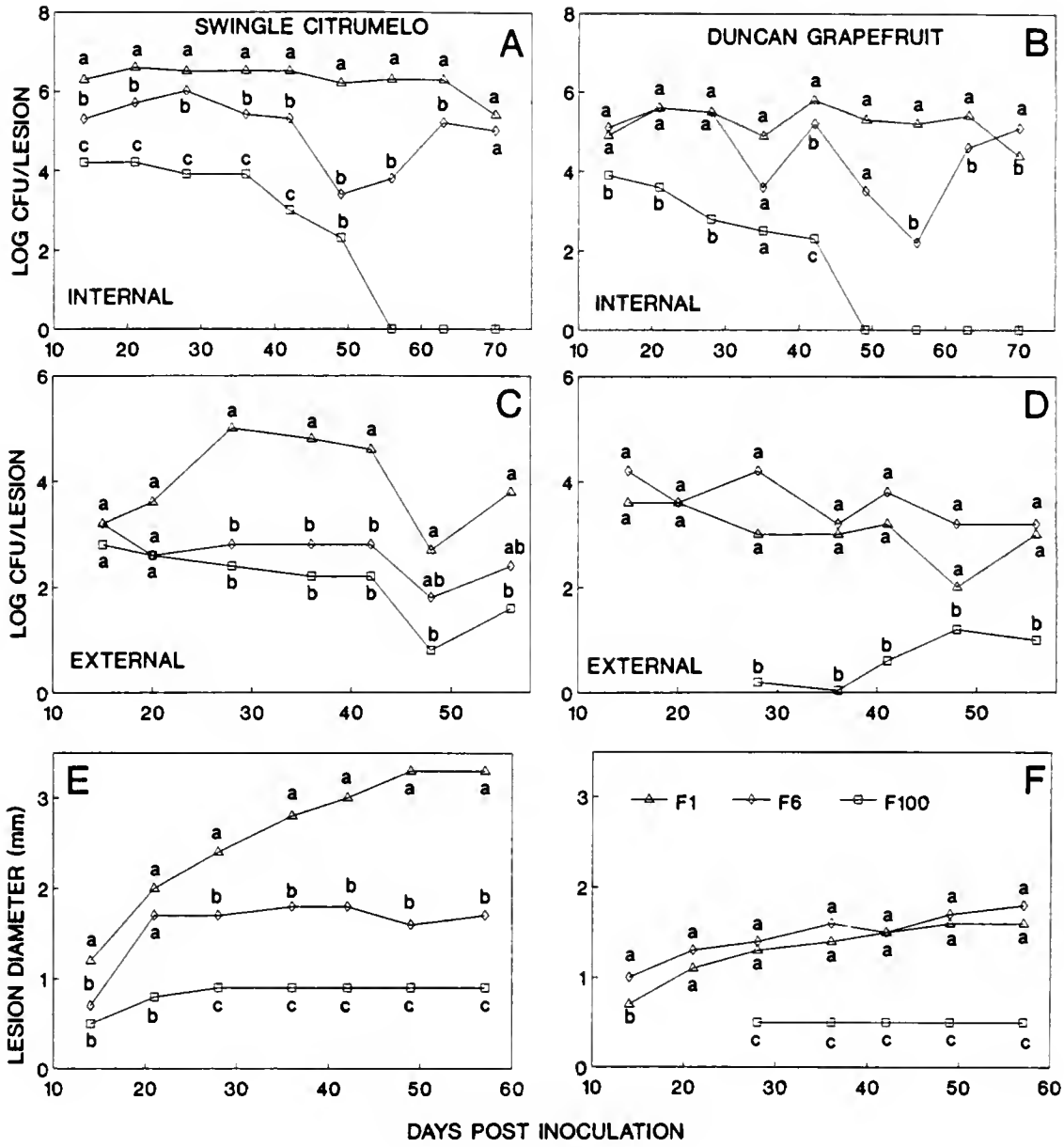


Figure 3-2. Internal (A) and external (B) leaf populations of Xanthomons campestris pv. citri strain MF23P and X. c. citrumelo strains F1, F6, and F100 and expansion of lesions (C) on Swingle citrumelo seedlings inoculated by a pin-prick method under growth chamber conditions. Each data point is the mean of five replications. Mean values on each sampling date are significantly different from means for treatments on that same date that are not accompanied by the same letter by Tukey's HSD, α=0.05.

Figure 3-3. Internal and external populations of Xanthomonas campestris pv. citrumelo strains F1, F6 and F100 and expansion of lesions on Swingle citrumelo (A, C, E) and Duncan grapefruit (B, D, F) leaves inoculated by a wounding method in field nurseries. Each data point is the mean of seven seedlings. Mean values on each sampling date are significantly different from means for treatments on that same date that are not accompanied by the same letter by Tukey's HSD, $\alpha=0.05$.


SWINGLE CITRUMELO
A
INTERNAL
DUNCAN GRAPEFRUIT
B
INTERNAL
C
EXTERNAL
D
EXTERNAL
E
F
F1
F6
F100
LOG CFU/LESION
LOG CFU/LESION
LESION DIAMETER (mm)
DAYS POST INOCULATION

cfu/lesion on Duncan grapefruit but were higher on Swingle citrumelo and similar to populations of F6 (Fig. 3-3C, 3-3D).

The ranking of aggressiveness types by lesion expansion after 56 days (Fig. 3-3E, 3-3F) corresponded with rankings by bacterial populations levels in and on lesions. Lesions produced by strain F1 on Swingle citrumelo continued to expand up to 49 days and the final lesion diameter far exceeded that on other host-strain combinations (Fig. 3-3E, 3-3F). These watersoaked lesions sometimes coalesced, producing leaf abscission and stem dieback. In contrast to strain F1, lesions elicited by strain F6 and F100 appeared dry and stopped expanding after 20 days on both hosts. On Duncan grapefruit, lesions produced by strain F100 did not appear until after 28 days and did not expand thereafter.

When all three X. campestris pv. citrumelo strains were considered, lesion diameter at 41 days on Swingle citrumelo and Duncan grapefruit was highly correlated with both internal and external populations on lesions (Table 3-1). Internal and external populations were also well correlated with each other. The correlations derived from field were higher than for the same comparisons on Swingle citrumelo in the growth chamber.

Discussion

Strains of X. campestris pv. citrumelo varied in external and internal populations and lesion diameters

produced upon wound inoculation of Swingle citrumelo and Duncan grapefruit in simulated nurseries. The highly aggressive strain F1 on Swingle citrumelo produced higher external and internal populations and lesion diameters than all other strain-cultivar combinations in the field. These results confirm and extend similar greenhouse experiments in which the highly aggressive F1 strain had higher populations in lesions on Swingle citrumelo and its parent trifoliate orange than on other citrus cultivars (Graham et al., 1990a). In addition, external and internal populations and lesion diameters were strongly correlated for the different aggressiveness types of X. campestris pv. citrumelo in the field, although less so in the growth chamber. These findings explain why the highly aggressive strains of X. campestris pv. citrumelo appeared to be spread by wind driven rain in field situations, but the less aggressive strains probably spread only by mechanical means (Gottwald and Graham, 1990; Gottwald and Graham, unpublished data). These data strengthen the contention that, among the aggressiveness types, the highly aggressive strains are the only pathogens of Swingle citrumelo and related cultivars that should be classified as X. campestris pv. citrumelo (Graham et al., 1990c).

Leaf surface populations, whether from lesions as in this study or living epiphytically, serve as an important source of inoculum for pathogen spread (Crosse, 1959;

Crosse, 1963; Crosse, 1966; Crosse and Bennett, 1955; Ercoloni et al., 1974). Such external populations have been related to internal populations for Xanthomonas campestris pv. phaseoli (Cafati and Saettler, 1980b) and Pseudomonas phaseolicola (Stadt and Saettler, 1981) on beans. Monitoring internal populations in leaf lesions may be used to determine the potential of a pathogen to spread and may serve as an alternative to estimating leaf surface populations which are highly variable and more difficult to sample.

Using more artificial inoculation methods under greenhouse or growth chamber conditions, there were not clear population differences between X. campestris pv. citri group A and X. campestris pv. citrumelo or among the X. campestris aggressiveness types. The internal populations of injection-infiltrated leaves were probably not an accurate indication of the capability of these strains to develop in lesions and become available for spread in the field. For example, lesions produced by the highly and moderately aggressive strains were indistinguishable on Swingle citrumelo after injection-infiltration. Injection into the leaf mesophyll may deliver bacteria to many susceptible sites over a large area. In contrast, by inoculating with a wounding technique, population growth depended on lesion expansion from a relatively small point of introduction. The pin-prick technique was demonstrated

previously to be more effective than either leaf spray or injection of X. campestris pv. citrumelo to determine susceptibility of citrus cultivars (Garran, 1988), and to determine the host-strain interaction of X. campestris pv. citrumelo strains (Graham and Gottwald, 1990; Graham et al., 1990a).

Likewise, the highly conducive dew-forming conditions in the growth chamber were inappropriate for distinguishing leaf surface populations on Swingle citrumelo of highly aggressive strain populations from the less aggressive strains. O'Brien and Lindow (1989) found that differences in epiphytic populations were more likely to be demonstrated with wetting and drying cycles than in constantly humid conditions. In these growth chamber experiments, humidity was maintained at 92% or higher, which probably accounted for the lack of differences in leaf surface populations of the highly and moderately aggressive strains on Swingle citrumelo. Field conditions of wetting and drying cycles more truly demonstrated the greater potential for the highly aggressive strains to produce bacteria on leaf surfaces of Swingle citrumelo than in any other strain-cultivar combination.

Furthermore, under field conditions it was possible to ascertain whether strains of X. campestris pv. citrumelo behave as true epiphytes in the absence of internal populations. Internal populations of the weakly aggressive strain F100 were not detectable by day 56 and day 49 on

Swingle citrumelo and Duncan grapefruit respectively, whereas external populations of F100 were detected beyond this time. Sampling by absorbing moisture off of lesions was designed to detect bacteria under conditions conducive for exudation and survival, i.e., mornings when dew formed an excellent microclimate for bacterial activity on leaves. Sampling of lesions for internal populations was conducted at midafternoon, when leaves were dry and conditions inhospitable for bacterial survival at least on the leaf surface (Graham, unpublished data). Thus, bacterial populations which existed on the lesion surface were not detected when sampling for internal populations. The continued presence of F100 on leaf surfaces in the absence of internal populations may indicate that F100 is capable of an epiphytic existence independent of lesion populations (Leben, 1981). Capability for epiphytic survival by X. campestris pathovars has been demonstrated on host and non-host plants in the absence of disease (Cafati and Saettler, 1980a; Mulrean and Schroth, 1982; Timmer, et al., 1987; Wrather et al., 1986). Multiplication of strains of X. campestris pv. citrumelo on leaf surfaces may have played a role as an inoculum source for the original outbreaks of citrus bacterial spot in citrus nurseries where no obvious source of bacteria was observed.

CHAPTER 4
GENOMIC CHARACTERIZATION

Asiatic citrus canker, caused by Xanthomonas campestris pv. citri, is world wide in distribution and has been known since at least 1899 (Tanaka, 1918). In 1984, strains of X. campestris were isolated from leafspots in a central Florida citrus nursery which seemed to cause a different disease (Schoulties et al., 1987). This disease has been designated Citrus bacterial spot (Graham and Gottwald, 1988), and the causal organism named X. campestris pv. citrumelo (Gabriel et al., 1989).

Several methods have been used in an effort to characterize these two pathovars and the relationship between them. Restriction endonuclease analysis of frequently occurring recognition sites revealed that strains of X. campestris pv. citrumelo are heterogeneous and distinct from strains of X. campestris pv. citri group A which are relatively homogeneous (Hartung and Civerolo, 1987). These relationships were confirmed and quantified by isozyme (Kubicek et al., 1989) and restriction fragment length polymorphism (RFLP) analyses (Gabriel et al., 1988, 1989; Hartung and Civerolo, 1989, Graham et al., 1990c). Similarity values of RFLPs were relatively low among weakly

aggressive strains of X. campestris pv. citrumelo (Graham et al., 1990c). Based on these similarity values and weak pathogenicity of many of these strains, it was suggested that some strains of X. campestris pv. citrumelo had been only incidentally isolated from citrus (Gabriel et al., 1989; Graham et al., 1990c). In addition, several pathovars of X. campestris were found to be genetically related to strains of X. campestris pv. citrumelo, and pathogenicity tests indicated an overlap of host ranges (Gabriel et al., 1988, 1989; Graham et al., 1990c; Hartung and Civerolo, 1989). Finally, on the basis of RFLP data, Gabriel et al. (1989) proposed to elevate X. campestris pv. citri group A to species status and to separate Florida nursery strains into X. campestris pv. citrumelo.

Although the above studies have yielded a level of understanding of the variability among these bacteria, the strains involved have yet to be compared using DNA reassociation. Reassociation of DNA is a standard technique which has been useful in estimating genetic distance between bacterial strains (Grimont, 1988). Many of these strains have not been previously described and their taxonomic status is uncertain; it has been recommended that final taxonomic designations include DNA reassociation analysis (Wayne et al., 1987).

The presence of several published RFLP analyses on strains of X. campestris pv. citri and X. campestris pv. citrumelo (Gabriel et al., 1988, 1989; Graham et al., 1990c;

Hartung and Civerolo, 1989) presents an excellent opportunity to compare conventional RFLP analyses to restriction endonuclease analysis of infrequently occurring recognition sites in genomic DNA fragments separated by pulsed field gel electrophoresis (Cooksey and Graham, 1989; Grothues and Tümmler, 1987; Le Bourgeois et al., 1989; Sorbral et al., 1990; Tanskanen et al., 1990). Restriction endonuclease analysis has been useful in discriminating between closely related strains of Pseudomonas syringae pv. tomato and X. campestris pv. vesicatoria (Cooksey and Graham, 1989). In this study, restriction endonuclease patterns were used to analyze diversity within groups of closely related strains.

By using DNA reassociation and restriction endonuclease analysis, the genetic distance among and between strains of X. campestris pv. citri and X. campestris pv. citrumelo was determined. In addition, the genetic distance between the above pathovars and other X. campestris pathovars, especially those pathovars or strains which form lesions on citrus was of interest. The final objective was to compare similarities generated by DNA reassociation and restriction endonuclease analysis to published similarities generated by other techniques.

Materials and Methods

Culture conditions. The taxonomic designations and sources of bacterial strains are listed in Table A-1. Before use, all strains were streaked onto nutrient agar

(Difco, Detroit, MI) or Lima bean agar (Difco) and single colonies selected. Nutrient broth cultures were grown 12-16 hours on a circular shaker (150 rpm) at 30 C. Strains of X. campestris pv. citri group B were grown in a sucrose based medium (Canteros de Echenique et al., 1985). Long term storage of bacteria was in nutrient broth/glycerol (85/15%, v/v) at -70 C.

DNA isolation. A procedure modified from Boucher et al. (1987) was used to extract DNA for DNA reassociation experiments. Bacterial cells from 300 ml nutrient broth were pelleted by centrifuging 10 min at 8000 g. The resulting pellet was washed in 40 ml TE8 (50 mM Tris, pH 8.0; 20 mM EDTA, pH 8.0), pelleted again and resuspended in 15 ml TE8. Deoxynucleases were inactivated by incubating the cells at 70 C for 15 min. After allowing the preparations to cool to room temperature, Proteinase K (Boehringer Mannheim Indianapolis, IN) and N-lauryl sarkosyl (Sigma, St. Louis, MO, sodium-salt) were added for a final concentration of 200 μg/ml and 0.5%, respectively. After incubation at 50 C for at least 15 hours, preparations were removed from the water bath and allowed to cool to room temperature. Each preparation was made to 2 M ammonium acetate. Five mls of phenol(Fisher):chloroform:isoamyl-alcohol (25:24:1) was added, the preparations were shaken by hand for 10 min and centrifuged 10 min at 8000 g. The aqueous phase was removed and the extraction repeated until

there was no protein interface (at least 3X). DNA was precipitated by the addition of 2 volumes of cold 95% ethanol and spooled with a heat sealed pasteur pipet. After allowing the DNA to air dry a few minutes, the DNA was dissolved in $T_{10}E_1$ (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0). The removal of RNA was accomplished by adding 500 μg RNase A (Sigma, type III-A) and 500 units RNase T_1 (Sigma) to each 10 ml preparation and incubating at 37 C for 60 min. After RNase treatment, 5 ml of a chloroform:isoamyl alcohol mixture (24:1) was added to each preparation which was shaken 10 min by hand and centrifuged as above. This step was repeated as needed. The aqueous layer was removed and the DNA precipitated as above. The DNA was dissolved in 0.1 X SSC (15 mM sodium chloride; 1.5 mM sodium citrate) and adjusted spectrophotometrically (260 nm) to 400 μg/ml and sheared by passing 3X through a French Press (16,000 psi).

DNA reassociation. The experimental procedure described herein has been adapted from Johnson (1985). Sheared DNA was radiolabelled using the random primed method according to instructions of the manufacturer (Boehringer Mannheim) to incorporate tritiated dCTP into genomic DNA. Reassociation reaction mixtures consisted of 10 μl denatured, labelled DNA (approx. 0.02 μg), 50 μl of denatured, unlabelled DNA (20 μg), 25 μl of salt buffer (5.15 M NaCl, 3 mM HEPES buffer, pH 7.0, final concentration equivalent to 6 X SSC) and 25 μl of formamide (Boehringer

Mannheim, electrophoretic grade) (final concentration, 22.7%, deionized by mixing with amberlite MB-3 resin, 10 % w/v) added to 500 µl microcentrifuge tubes. In this fashion, radiolabelled DNA from one strain was compared to DNA of several strains of interest. Each experiment included replicate samples of each comparison and four replicate homologous controls (labelled and unlabelled DNA from the same strain) and four controls to measure self-reassociation of labelled DNA using sheared, native, herring sperm DNA (Boehringer Mannheim) for the unlabelled DNA.

The DNA was allowed to reassociate for 20 hrs submerged in a 57 C water bath. At the end of this period, 24 µg of sheared, denatured herring sperm DNA and 300 units of S1 nuclease (Bethesda Research Laboratories, Bethesda, MD) were added to each tube, and the tubes incubated in a 50 C water bath for 1 hour to eliminate single stranded DNA. Double stranded DNA was precipitated by adding 30 µg sheared, native, herring sperm DNA and 1/5 volume of ice cold acid solution (1 N HCl, 10% (w/v) sodium pyrophosphate and 10% (w/v) momobasic sodium phosphate) and incubating on ice 1 hr.

The temperature of 57 C for reassociation experiments was derived from the midpoint of the mol % GC range for the species X. campestris (63.5-69.2, Bradbury, 1984). The midpoint, 66.4, can be converted into a Tm value (96.5 C) using Marmur's equation (Marmur and Doty, 1962) (Tm=69.3 +

0.41[% GC content]). Standard reassociation experiments are conducted 25 C below Tm (Johnson and Ordall, 1968), so 96.5 C - 25 C=71.5 C. This temperature is lowered 0.7 (Maniatus et al., 1982) or 0.61 (Johnson, 1985) degrees for each 1% formamide used; therefore the final figure is 57 C (using a value midway between 0.7 and 0.61 degrees for each percent formamide).

Labelled double stranded DNA was quantified using a method modified from Preston et al. (1975). After precipitation, the reassociated, double stranded DNA was separated from unincorporated nucleotides by filtering through Whatman GF/C glass fiber filters at 15 psi vacuum. Each sample was washed 3X with 10 to 15 ml of the acid solution and subsequently 3X with 10 to 15 ml 95% ethanol. The vacuum was maintained for an additional 10 minutes to dry the filters. Additional drying was accomplished by incubating the filters in an oven at 50-55 C overnight. Filters were placed in 5 ml scintillation cocktail and radioactivity quantified by liquid scintillation counting.

Each sample contained 0.13 μci of tritiated dCTP incorporated into linear genomic DNA. This level of radioactivity usually generated between 20,000 and 30,000 cpm for the homologous controls and 10 to 20% of this value for reassociation controls. Heterologous controls ranged between these values depending on the similarity of the DNA being compared. Counting efficiency was 17%.

Percent similarities were calculated by first

subtracting reassociation control values from all samples and then calculating the percent of each comparison from the homologous value. All values herein represent the mean of at least two experiments. The similarity values generated by each experiment were considered a replication and standard errors were calculated based on these replications.

Restriction endonuclease analysis. The methods used were similar to those reported earlier (Cooksey and Graham, 1989). Each strain was grown for 12-16 hrs at 30 C at 150 rpm on a rotary shaker in nutrient broth inoculated from a single colony. Cells were pelleted in a Beckman micro-centrifuge, washed once in SE buffer (NaCl 75 mM, EDTA 25 mM, pH 8.0) with the cells finally resuspended in 0.5 ml SE buffer. The cell suspension was mixed with 0.5 ml melted, cooled, low melting point agarose solution (10 mM Tris, pH 8.0; 10 mM $MgCl_2$; 0.1 mM EDTA, pH 8.0; 2% w/v LMP agarose, Bethesda Research Laboratories, in sterile distilled water) and pipetted into a Bio-rad (Richmond, CA) plastic mold. The mold was then placed at 4 C for 10-15 min. After the agarose had hardened, the inserts were removed from the mold and transferred to lysing solution (0.5 mg/ml Proteinase K; 1% w/v N-lauryl sarkosyl; 0.5 mM EDTA, pH 9.5) in sterile tubes. The tubes were placed in a 50 C water bath and the cells lysed overnight (at least 15 hrs).

After lysis, the inserts were removed from the lysis solution and placed in sterile TE buffer. After 15 min at

room temperature, the TE solution was changed and the inserts incubated for an additional 6-8 hrs. The inserts were then removed from the TE buffer and a 1-2 mm slice cut from the insert and placed in a microcentrifuge tube with 200 μl restriction buffer (as obtained from the manufacturer, Boehringer Mannheim); the remainder of the insert was saved in 250 mM EDTA, pH 8.0, at 4 C. After 15 min incubation at room temperature, the restriction buffer was changed and 30 units of either Xba I or Spe I (Boehringer Mannheim) restriction enzyme was added. Microcentrifuge tubes were then incubated at least 8 hrs at 37 C in a horizontal position. After incubation, the restriction buffer was removed and 500 μl lysing solution (without proteinase K) was added. Samples were incubated at 50 C in a water bath for 2 hrs, the lysing solution changed, and the samples incubated for an additional 2 hrs at room temperature.

The agarose slices were then ready for electrophoresis. The slices were placed in the wells of a 1% gel made with 0.5 X TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) and the wells sealed with cooled 1% agarose. The gel was placed in a BIO-RAD CHEF DR II (Chu et al., 1986) unit containing approximately 1.6 L of 0.5 X TBE and run at 200 volts (16 V/cm gel). Unless otherwise indicated, pulse times for DNA restricted with Xba I were 4 sec for 2 hr and 15 sec for 22 hrs and for DNA restricted with Spe I pulse

times were increased linearly from 4 sec to 50 sec for 22 hrs. Phage λ concatemers from BIO-RAD were used as molecular markers. Gels were stained in 0.5 mg ethidium bromide per liter and photographed with type 55 Polaroid film.

Similarity values were calculated as follows. The number of fragments greater than 100 kb shared between strains was recorded for strains on the same gel. The similarity coefficient was calculated using the following formula:

$$S=2n_{xy}/(n_x + n_y),$$

where n_{xy} was the number of fragments shared between two strains and n_x and n_y were the total number of fragments larger than 100 kb for strain x and strain y, respectively.

Comparisons between methods. A summary of the results presented here was compared with published accounts dealing with the same pathovars or groups of strains. The published reports used for comparison purposes were: Gabriel et al., 1988, 1989; Graham et al., 1990c; Hartung and Civerolo, 1989; and Kubicek et al., 1989. Several investigators recognized three groups within X. campestris pv. citrumelo based on aggressiveness and genetic relatedness (Gabriel et al., 1989; Graham et al., 1990c; Hartung and Civerolo, 1989). These groups were called, respectively, highly, moderately and weakly aggressive by Graham et al. (1990c); E2, E1 and an unspecified group by Gabriel et al. (1989);

and subgroups E, F and G by Hartung and Civerolo (1989). The similarity values for all comparisons in which both strains were within a particular group (within group comparisons) were averaged and the standard deviation calculated for each applicable investigation. Also presented is the overall similarity for all comparisons where both strains were X. campestris pv. citrumelo, again for each applicable investigation. In the same manner, similarity values for comparisons in which both strains were either X. campestris pv. citri group A or B were averaged and the standard deviation calculated for each applicable data set. Similarities for between group comparisons are also presented. Similarity values for all relevant comparisons were averaged and the standard deviation calculated.

Results

DNA reassociation. The strains which comprise X. campestris pv. citrumelo were highly related to one another by DNA reassociation (Table 4-1). Aggressiveness groups of X. campestris pv. citrumelo (Graham and Gottwald, 1990) were not distinguished by DNA reassociation; all strains were ca. 80-90% related, however, there were two comparisons of 76% (Table 4-1). It is of interest to note that both comparisons involved strain F100.

Strains of X. campestris pv. citrumelo representing all three aggressiveness types were ca. 60% related to strain

9771 of X. campestris pv. citri group A (Table 4-2). Similarly, both strain 9771 and strain F1 of X. campestris pv. citrumelo were ca. 60% related to the two strains of X. campestris pv. citri group B (Table 4-2). Therefore, bacterial strains representing three different diseases of citrus were only distantly related to each other. Strain 33913 of X. campestris pv. campestris, the type strain of X. campestris, was 34% related to strain F1 of X. campestris pv. citrumelo, ca. 40% related to strains B64 and B94 of X. campestris pv. citri group B and 30% similar to strain 9771 of X. campestris pv. citri group A.

In an attempt to place X. campestris pv. citri and X. campestris pv. citrumelo within the context of noncitrus pathovars of X. campestris, strains 9771 and F1 were compared to several pathovars. Strains of X. campestris pv. campestris, X. campestris pv. vesicatoria, and X. campestris pv. phaseoli were not closely related to either strain 9771 or strain F1 (Table 4-3). Strains Xv 56 and Xv 75-3 of X. campestris pv. vesicatoria each gave different relatedness values to strain 9771 and F1. Strain N of X. campestris pv. malvacearum was only 71% similar to strain F1, but was 90% related to strain 9771. Strain Xp 20 of X. campestris pv. phaseoli was ca. 40% distant to strains 9771 and F1. Type strain 33913 of X. campestris pv. campestris was not closely related to any of the strains in these experiments.

Four strains of X. campestris, not isolated from citrus but capable of lesion formation on citrus, were compared to

strains of the three aggressiveness types of X. campestris pv. citrumelo (Table 4-4). Strain X198 of X. campestris from Strelitzia reginae, strain X151 of X. campestris pv. fici and strain 82-1 of X. campestris pv. alfalfae ranged from 80 to 91% related to the strains of X. campestris pv. citrumelo. However, strain X22j of X. campestris pv. maculifoliigardeniae ranged from 68 to 76% related to aggressiveness types of X. campestris pv. citrumelo.

Restriction endonuclease analysis. Strains which shared common pathogenicity traits were compared on the same gel. The four pathogenicity groups analyzed here include two aggressiveness types of X. campestris pv. citrumelo (moderately and weakly aggressive), and X. campestris pv. citri groups A and B. The highly aggressive strains of X. campestris pv. citrumelo could not be compared by restriction endonuclease analysis due to excessive shearing of genomic DNA prior to or during lysis. This problem was also encountered with strain F100 of the weakly aggressive group.

Seven moderately aggressive strains had few comigrating DNA fragments when restricted with Xba I or Spe I (Figs. 4-1 and 4-2). The weakly aggressive strains restricted with Xba I had similarly diverse restriction patterns (Fig. 4-3).

In contrast to the diversity of the moderately and weakly aggressive strains of X. campestris pv. citrumelo, X. campestris pv. citri groups A and B each gave characteristic restriction patterns (Figs. 4-4, 4-5, 4-6, 4-7). The

restriction patterns of four of the strains of X. campestris pv. citri group A were identical to each other as restricted by Xba I and Spe I (Fig. 4-4 and 4-5, Table 4-5), however strain T1 exhibited one polymorphism with each restriction endonuclease reducing its similarity to the other strains.

Although not as homogenous as X. campestris pv. citri group A from Florida, the strains of X. campestris pv. citri group B are moderately to highly related as seen in Xba I and Spe I restriction patterns (Figs. 4-6 and 4-7). These strains ranged from 0.57 to 0.98 similarity when Xba I and Spe I derived values were averaged (Table 4-6).

Comparisons between methods. Similarity values for comparisons within X. campestris pv. citrumelo and X. campestris pv. citri groups A and B varied by technique and by investigation. However, the similarity values for comparisons among highly aggressive strains of X. campestris pv. citrumelo and among strains of X. campestris pv. citri group A or X. campestris pv. citri group B tended to be similar based on all techniques (Table 4-7). Similarity values for moderately and weakly aggressive strains of X. campestris pv. citrumelo were generally higher based on DNA reassociation than for RFLP analyses. Overall similarities for X. campestris pv. citrumelo comparisons were higher for DNA reassociation and Hartung and Civerolo (1989) RFLP analysis than for Gabriel et al. (1989) RFLP or isozyme analysis (Kubicek et al. (1989). In general, similarity

Table 4-1. Similarity values generated by DNA reassociation for strains of Xanthomonas campestris pv. citrumelo causing citrus bacterial spot.

Aggressiveness Type[a]	Strain	F1	F6	F100
Highly	F1	100[b]	--	--
aggressive	F54	91 (0.1)[c]	ND[d]	ND
	F274	84 (6.9)	ND	ND
	F361	91 (2.8)	ND	ND
Moderately				
aggressive	F6	89 (0.9)	100	--
	F228	86 (4.4)	96 (4.0)	ND
	F254	102 (16.0)	82 (6.2)	ND
	F311	80 (2.3)	89 (6.2)	ND
	F348	91 (8.1)	84 (4.5)	ND
	F397	90 (18.9)	87 (3.3)	ND
Weakly				
aggressive	F59	85 (4.2)	87 (4.9)	83 (5.9)
	F86	99 (11.4)	92 (4.0)	82 (6.3)
	F94	96 (13.3)	89 (4.5)	92 (6.6)
	F100	87 (3.2)	76 (7.6)	100
	F306	100 (7.9)	90 (0.5)	76 (4.9)

[a]Strains F1, F6 and F100 represent, respectively, highly, moderately and weakly aggressive strains of X. campestris pv. citrumelo as rated by a detached leaf assay (Graham and Gottwald, 1990).
[b]Value represents percentage of homologous value which was set to 100%.
[c]standard error of the mean.
[d]Not Done.

Table 4-2. Similarity values generated by DNA reassociation for strains of Xanthomonas campestris causing diseases of citrus.

	X. c. pv. campestris	X. c. pv. citri		
	33913[T]	B64[a]	B94[a]	9771[a]
F1[b]	34 (0.3)[c]	59 (6.6)	57 (4.8)	56 (3.3)
F6[b]	ND[d]	ND	ND	55 (6.4)
F100[b]	ND	ND	ND	61 (6.3)
9771	30 (4.2)	63 (1.8)	62 (0.1	100[e]
33913[T]	100	37 (8.0)	40 (8.7)	--

[T]Type strain 33913 is the type strain of X. campestris and causes black rot of crucifers.
[a]Strains B64 and B94 are X. c. pv. citri group B and cause Cancrosis B; Strain 9771 is X. c. pv. citri group A and causes Asiatic citrus canker;
[b]Strains F1, F6 and F100 are, respectively, highly, moderately, and weakly aggressive strains of X. c. pv. citrumelo and cause Citrus bacterial spot.
[c]standard error of the mean.
[d]Not Done
[e]Value represents percentage of homologous value which was set to 100%.

Table 4-3. Similarity values generated by DNA reassociation for comparisons of *Xanthomonas campestris* pv. *citrumelo*, *X. campestris* pv. *citri* and *X. campestris* pv. *campestris* to *X. campestris* pathovars *vesicatoria*, *phaseoli* and *malvacearum*.

	Xanthomonas campestris pathovars			
	vesicatoria		*phaseoli*	*malvacearum*
	Xv 75-3	Xv 56	Xp 20[H]	N
F1[a]	58 (8.4)[b]	25 (1.9)	43 (0.8)	71 (8.8)
9771[c]	37 (4.8)	15 (7.6)	45 (5.8)	90(16.5)
33913[T]	21 (3.5)	18 (3.0)	36 (0)	36 (8.1)

[H]Holopathotype
[a]*X. campestris* pv. *citrumelo*, highly aggressive.
[b]standard error of the mean.
[c]*X. campestris* pv. *citri* group A
[T]Type strain of the species.

Table 4-4. Similarity values generated by DNA reassociation for strains of Xanthomonas campestris which produce lesions on citrus.

	Xanthomonas campestris pathovars			
	maculifolii-gardeniae X22j	undetermined X198[a]	fici X151	alfalfae 82-1
F1[b]	72 (4.1)[c]	89 (4.7)	90 (3.0)	88 (1.4)
F6[b]	68 (1.1)	86 (3.5)	91 (2.9)	80 (12.3)
F100[b]	76 (4.8)	90 (4.1)	87 (2.9)	83 (0.8)

[a]Isolated from Strelitzia reginae.
[b]Strains F1, F6 and F100 are, respectively, highly, moderately and weakly aggressive strains of X. c. pv. citrumelo (Graham and Gottwald, 1990).
[c]standard error of the mean.

Table 4-5. Similarity matrix of strains of Xanthomonas campestris pv. citri group A, which cause Asiatic citrus canker, generated by restriction endonuclease analysis of infrequently occurring recognition sites in genomic DNA fragments separated by pulsed field gel electrophoresis.

	9771	3213	3340	9760	T1
9771	--	1.00[a]	1.00	1.00	0.90
3213	--	--	1.00	1.00	0.90
3340	--	--	--	1.00	0.90
9760	--	--	--	--	0.90
T1	--	--	--	--	--

[a]Values are the average of similarities derived with Xba I and Spe I.

Table 4-6. Similarity values of strains of Xanthomonas campestris pv. citri group B, which cause Cancrosis B, generated by restriction endonuclease analysis of infrequently occurring recognition sites in genomic DNA fragments separated by pulsed field gel electrophoresis.

	B84	B93	B148	B80	B64	B69	B94
B84	--	0.85[a]	0.98	0.71	0.54	0.78	0.73
B93	--	--	0.82	0.76	0.59	0.69	0.73
B148	--	--	--	0.68	0.56	0.82	0.70
B80	--	--	--	--	0.69	0.67	0.78
B64	--	--	--	--	--	0.52	0.60
B69	--	--	--	--	--	--	0.63
B94	--	--	--	--	--	--	--

[a]Values are the average of similarities derived with Xba I and Spe I.

Table 4-7. Within group comparisons of similarity values generated in this paper to similarities published elsewhere for the same or similar strains of Xanthomonas campestris pv. citrumelo and X. campestris pv. citri.

	DNA reassociation[a]	Pulsed field gel electrophoresis[b]	RFLP comparisons			Fatty acid profiles[f]	Isozymes[g]
			Gabriel et al.[c]	Hartung & Civerolo[d]	Graham et al.[e]		
X. c. pv. citrumelo							
Highly aggressive[h]	0.89 (0.04)[i]	--	0.93 (0.04)	0.97 (0.03)	--	--	--
Moderately aggressive	0.87 (0.04)	--	0.65 (0.15)	0.95 (0.05)	--	--	--
Weakly aggressive	0.83 (0.07)	--	0.45 (0.13)	0.77 (0.02)	0.70 (0.14)	0.64 (0.20)	--
Overall Mean	0.88 (0.06)	--	0.51 (0.18)	0.81 (0.11)	--	--	0.68 (0.15)

Table 4-7--continued.

	DNA reas-soci-ation[a]	Pulsed field gel electro-phoresis[b]	RFLP comparions Gabriel et al.[c]	Hartung & Civerolo[d]	Graham et al.[e]	Fatty acid profiles[f]	Iso-zymes[g]
X. c. pv. citri							
group A	--	0.97 (0.04)	0.92 (0.06)	0.89 (0.08)	--	--	0.85 (0.13)
group B	--	0.71 (0.11)	100 --	0.94 (0.05)	--	--	0.91 (0.04)

[a]DNA reassociation values, this study.
[b]pulsed field gel electrophoresis restriction endonuclease analysis, this study.
[c]Restriction Fragment-length Polymorphism analyses of strains of X. c. pv. citrumelo are presented in Gabriel et al., 1989 and of strains of X. c. pv. citri in Gabriel et al., 1988.
[d]presented in Hartung and Civerolo, 1989.
[e]presented in Graham et al., 1990c.
[f]fatty acid similarity data presented in Graham et al., 1990c.
[g]Isozyme data presented in Kubicek et al., 1989.
[h]The highly, moderately, and weakly aggressive groups are presented in Graham et al., 1990c, and are equivalent to, respectively, E2, E1 and an unspecified group in Gabriel et al., 1989, and subgroup E, F and G in Hartung and Civerolo, 1989.
[i]standard deviation of similarity values.

Table 4-8. Between group comparisons from similarity values generated in this paper to similarities presented elsewhere for the same or similar groups of strains of Xanthomonas campestris pv. citrumelo and X. campestris pv. citri.

	DNA reas-soci-ation[a]	RFLP comparisons		Iso-zymes[d]
		Gabriel et al.[b]	Hartung & Civerolo[c]	
ACC[e] vs. CBS[f]	0.58 (0.03)[g]	0.23 (0.05)	0.34 (0.02)	0.63 (0.11)
ACC vs. CB[h]	0.62 (0.01)	0.16 (0.04)	0.60 (0.01)	0.77 (0.07)
CB vs. CBS	0.58 (0.01)	0.31 (0.02)	0.30 (0.06)	0.58 (0.08)

[a]DNA reassociation values, this study.
[b]Restriction Fragment-Length Polymorphism analysis presented in D. W. Gabriel et al., 1988.
[c]presented in Hartung and Civerolo, 1989.
[d]Isozyme data presented in Q. B. Kubicek et al., 1989.
[e]Strains of X. campestris pv. citri group A causing Asiatic citrus canker.
[f]Strains of X. campestris pv. citrumelo causing Citrus bacterial spot.
[g]standard deviation of similarity values.
[h]Strains of X. campestris pv. citri group B causing Cancrosis B.

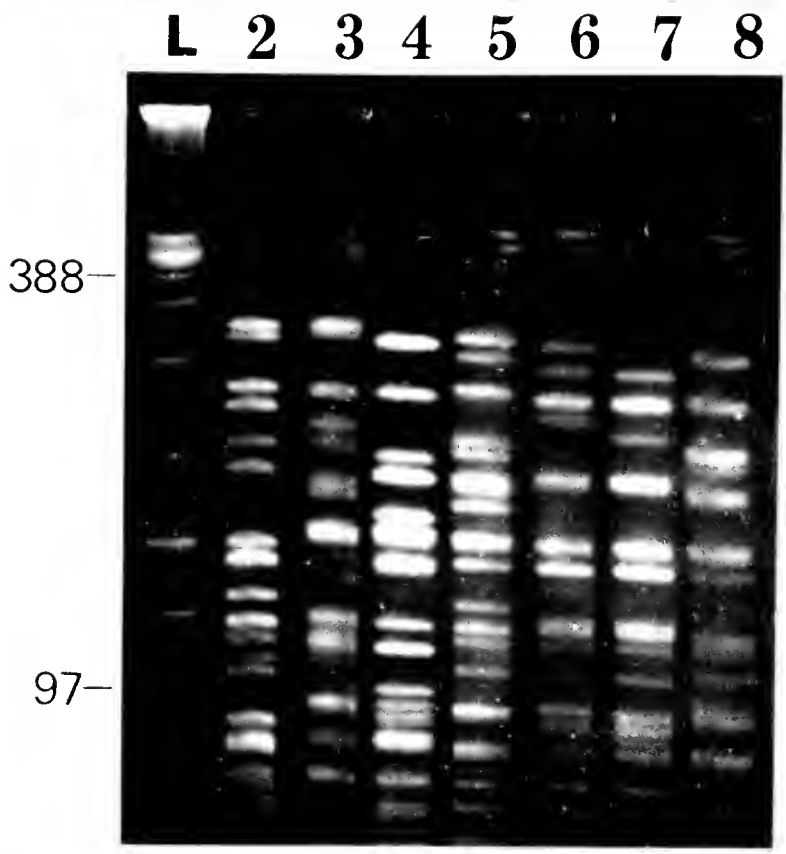


Figure 4-1. Restriction endonuclease patterns of moderately aggressive strains of Xanthomonas campestris pv. citrumelo restricted with Xba I. Electrophoresis was by Pulsed field for 1 hr at 4 sec and 22 hr at 15 sec. Lanes: L, phage λ concatamers; 2, F6; 3, F311; 4, F254; 5, F299; 6, 7222; 7, 3274; 8, 540. Molecular sizes are given in kilobases.

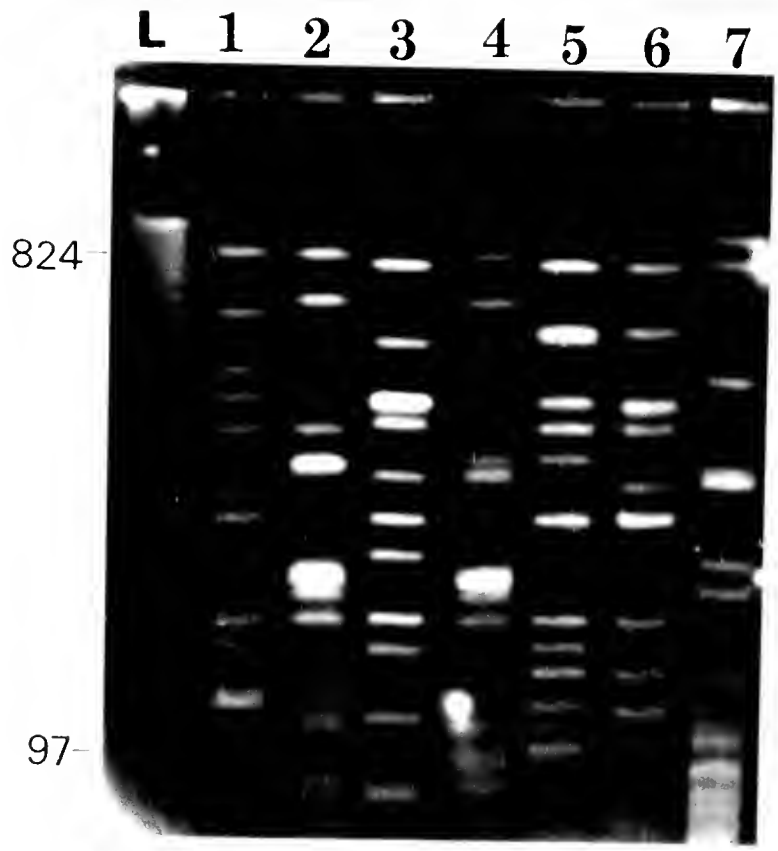


Figure 4-2. Restriction endonuclease patterns of moderately aggressive strains of Xanthomonas campestris pv. citrumelo restricted with Spe I. Electrophoresis was by Pulsed Field for 22 hrs with a pulse time increasing linearly from 4 to 50 sec. Lanes: L, phage λ concatemers; 1, F6; 2, F311; 3, 7222; 4, F254; 5, F299; 6, 3274; 7, 540. Molecular sizes are given in kilobases.

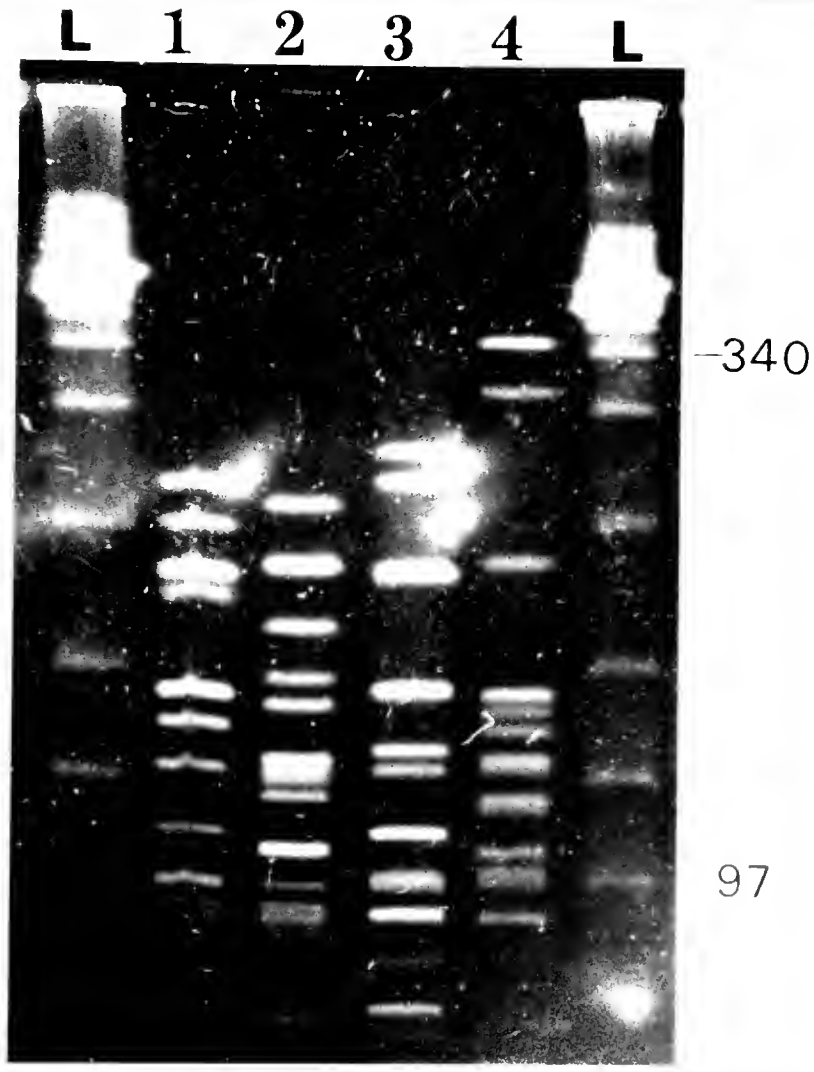


Figure 4-3. Restriction endonuclease patterns of weakly aggressive strains of Xanthomonas campestris pv. citrumelo restricted with Xba I. Electrophoresis was by Pulsed Field for 2 hr at 4 sec and 22 hr at 15 sec. Lanes: L, phage λ concatemers; 1, F306; 2, F59; 3, F94; 4, F86. Molecular sizes are given in kilobases.

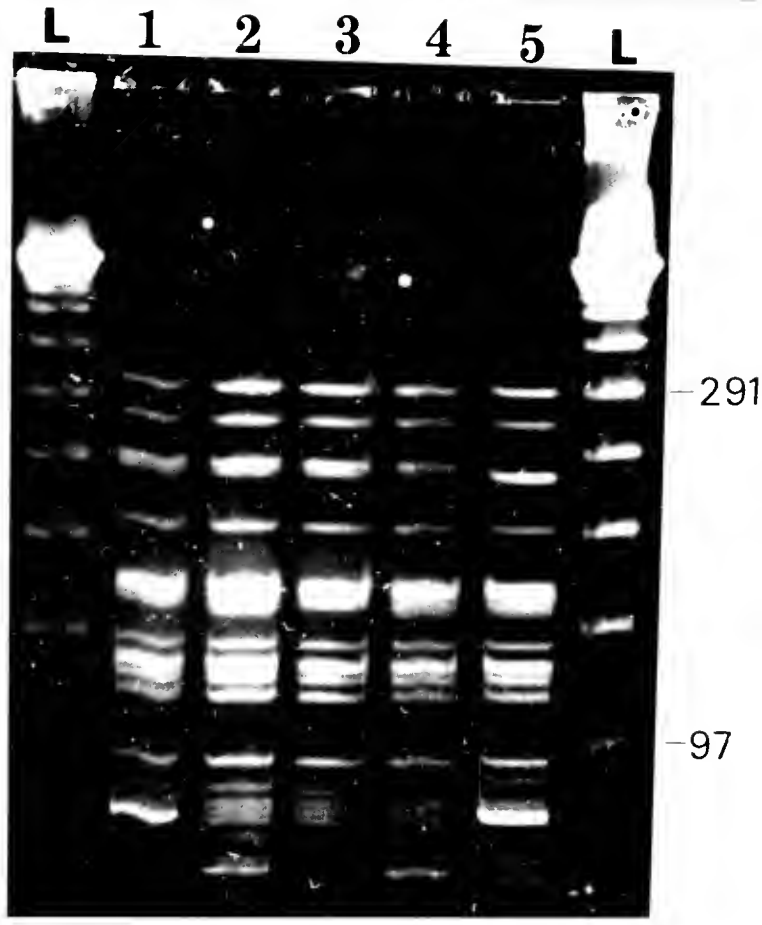


Figure 4-4. Restriction endonuclease patterns of strains of Xanthomonas campestris pv. citri group A restricted with Xba I. Electrophoresis was by Pulsed Field for 22 hr with a pulse time increasing linearly from 1 to 20 sec. Lanes: L, phage λ concatemers; 1, 9771; 2, 3213; 3, 9760; 4, 3340; 5, T1. Molecular sizes are given in kilobases.

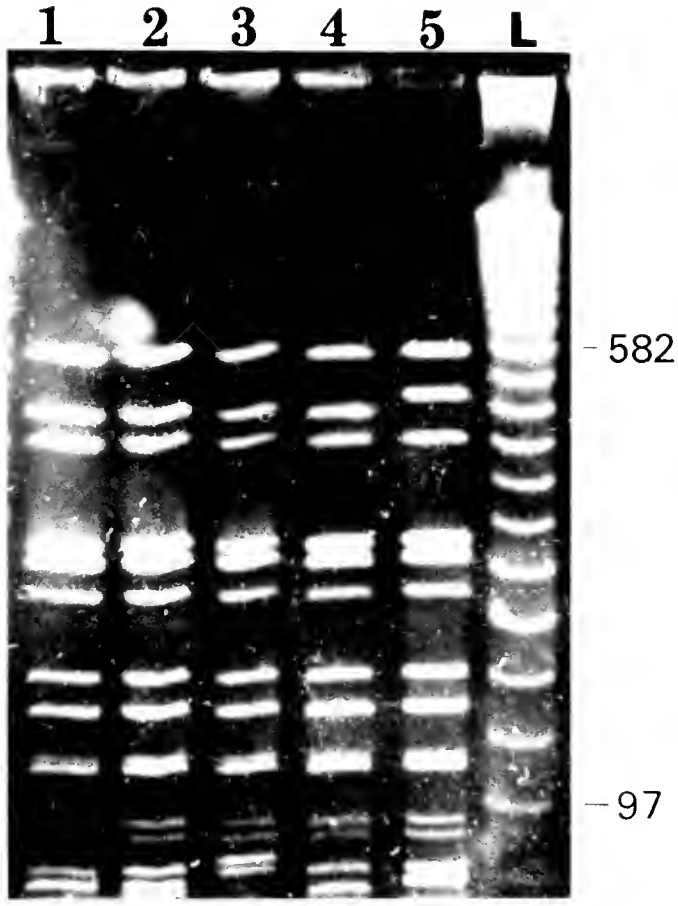


Figure 4-5. Restriction endonuclease patterns of strains of Xanthomonas campestris pv. citri group A restricted with Spe I. Electrophoresis was by Pulsed Field for 22 hr with a pulse time increasing linearly from 4 to 50 sec. Lanes: 1, 9771; 2, 3340; 3, 3213; 4, 9760; 5, T1; L, phage λ concatemers. Molecular sizes are given in kilobases.

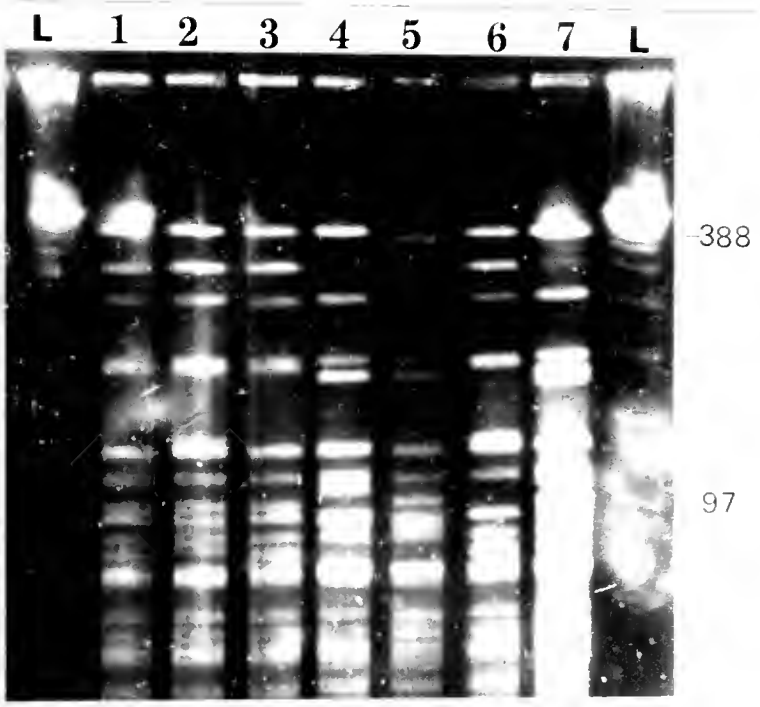


Figure 4-6. Restriction endonuclease patterns of strains of Xanthomonas campestris pv. citri group B restricted with Xba I. Electrophoresis was by Pulsed Field for 22 hr with a pulse time increasing linearly from 2 to 18 sec. Lanes: L, phage λ concatemers; 1, B80; 2, B93; 3, B64; 4, B69; 5, B148; 6, B84; 7, B94. Molecular sizes are given in kilobases.

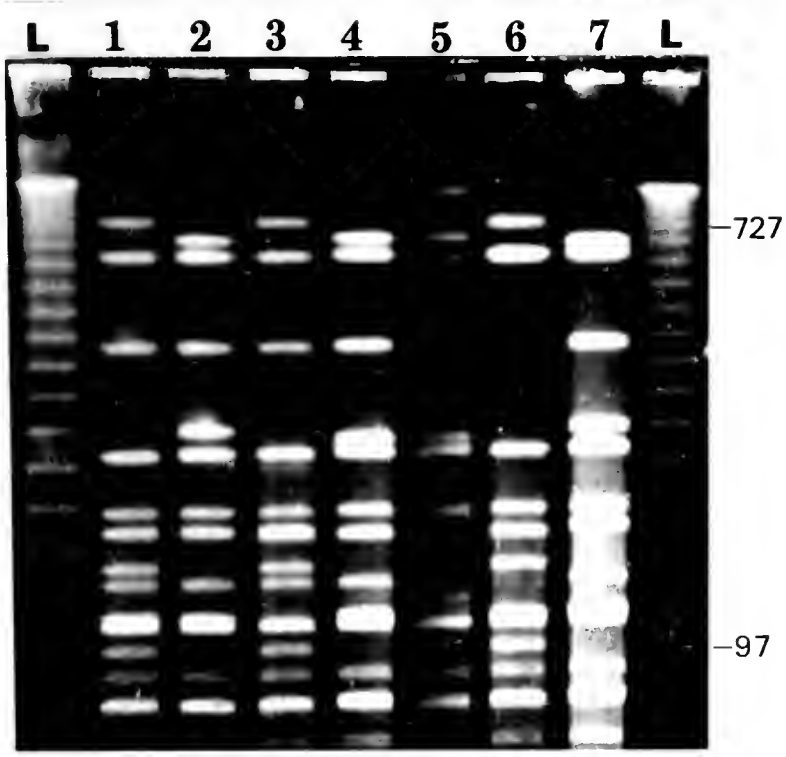


Figure 4-7. Restriction endonuclease patterns of strains of Xanthomonas campestris pv. citri group B restricted with Spe I. Electrophoresis was by Pulsed Field for 22 hr with a pulse time increasing linearly from 4 to 50 sec. Lanes: L, phage λ concatemers; 1, B84; 2, B93; 3, B148; 4, B80; 5, B64; 6, B69; 7, B94. Molecular sizes are given in kilobases.

values for Gabriel et al. (1989) RFLP analysis values were lower than similarities obtained by any other technique or investigator (Table 4-7).

Comparisons of similarity values for between pathovars are presented in Table 4-8. Similarity values for DNA reassociation were similar to isozyme values. When X. campestris pv. citri groups A and B were compared, DNA reassociation and Hartung and Civerolo (1989) RFLP values were both ca. 60%. In contrast, when X. campestris pv. citrumelo was compared to either X. campestris pv. citri group A or B, both sets of RFLP similarity values were lower than similarity values generated by DNA reassociation. Similarity values obtained by Gabriel et al. (1989) RFLP analysis were similar to Hartung and Civerolo (1989) RFLP values when X. campestris pv. citri group A was compared to X. campestris pv. citrumelo, but Gabriel et al. (1989) RFLP values were much lower than Hartung and Civerolo (1989) RFLP values (0.16 vs. 0.60, respectively) when strains of X. campestris pv. citri group A and B were compared.

Discussion

Although the bacterial strains responsible for Citrus bacterial spot, Asiatic citrus canker and Cancrosis B all belong to the species X. campestris and cause similar diseases on related hosts, X. campestris pv. citrumelo and the two groups of X. campestris pv. citri are only ca. 60% related to each other by DNA reassociation. The ability to

cause these similar diseases on citrus is either represented by a small portion of the genome and/or these pathogens have independently evolved means to cause similar diseases. These three groups of pathogens also differ in the diversity of strains within each group as revealed by restriction endonuclease analysis. The strains of X. campestris pv. citri groups A and B are closely related within each group, whereas, strains of X. campestris pv. citrumelo responsible for Citrus bacterial spot are more diverse.

Six DNA-DNA homology groups have been described within X. campestris, including group 1 which includes several legume pathovars and has similarity values of 50-90% (Vauterin et al., 1990a). Strain F1 of X. campestris pv. citrumelo may belong to DNA-DNA homology group 1 based on its similarity to strain Xv 75-3 of X. campestris pv. vesicatoria and strain Xp 20 of X. campestris pv. phaseoli. Strain 9771 of X. campestris pv. citri group A is similarly related to strain Xp 20, but is less related than strain F1 to strain Xv 75-3. Nevertheless, it is possible that both X. campestris pv. citrumelo and X. campestris pv. citri belong to DNA-DNA homology group 1.

Strain Xv 56 may belong to a separate X. campestris pv. vesicatoria group with little similarity to the Xv 75-3 group (Stall et al., unpublished) or any of the described DNA-DNA homology groups (Vauterin et al., 1990a). Little similarity was observed between X. campestris pv.

campestris, which belongs to a separate DNA-DNA homology group (Vauterin et al., 1990a), and any of the other strains analyzed here. Strain 9771 of X. campestris pv. citri was similar to X. campestris pv. malvacearum which was not included in any of the described DNA-DNA homology groups previously (Vauterin et al., 1990a). Fatty acid profiles of these two pathogens are also similar (Stall and Hodge, 1989), although X. campestris pv. malvacearum does not exhibit pathogenicity to citrus (Graham et al., 1990c).

Strain X198 of X. campestris, strain X151 of pathovar fici, and strain 82-1 of pathovar alfalfae have genomic similarities to X. campestris pv. citrumelo as shown by DNA reassociation data presented here as well as RFLP and fatty acid profiles (Graham et al., 1990c) and share the ability to cause lesions on citrus (Graham et al., 1990c). Collectively, these strains may form a group with a broad host range. If such a population exists, it may have implications for the origin of Citrus bacterial spot in Florida and the relatively diverse genetic nature of these pathogens.

As previously reported (Graham et al., 1990c), X. campestris pv. maculifoliigardeniae does not appear to be as related to X. campestris pv. citrumelo as strain X198 from Strelitzia sp. or X. campestris pv. fici. However, X. campestris pv. maculifoliigardeniae is capable of causing internal population growth and lesion development on citrus

(Graham et al., 1990c). As discussed above, ability to grow *in planta* in this pathosystem may constitute a small portion of the genome and/or may be superimposed on diverse genetic backgrounds.

The diverse nature of *X. campestris* pv. *citrumelo* has been emphasized previously (Gabriel et al., 1988, 1989; Graham et al., 1990c; Hartung and Civerolo, 1987; Hartung and Civerolo, 1989; Kubicek et al., 1989). Although the diverse nature of this group has been confirmed here with restriction endonuclease analysis, DNA reassociation similarity values within the moderately and weakly aggressive strains of *X. campestris* pv. *citrumelo* are considerably higher than previously published RFLP values (Gabriel et al., 1988, 1989; Graham et al., 1990c; Hartung and Civerolo, 1989). Similarly, in a characterization of pathovars of *Pseudomonas syringae*, DNA reassociation similarity values tended to be higher than corresponding RFLP values (Denny et al., 1988). Whereas similarities generated by RFLPs provide a relative measure of the genetic difference between strains, DNA reassociation is valuable in estimating the total genomic distance between bacterial strains. By combining both approaches, a more accurate picture of the relationships among strains emerges. The RFLP analyses indicate differences between aggressiveness types; however the DNA reassociation data is interpreted here to indicate that the total genetic distance among these

strains is relatively small. In contrast, highly related strains, e.g. of highly aggressive strains of X. campestris pv. citrumelo, had high similarities regardless of technique employed. Although diverse as illustrated with RFLP analyses and restriction endonuclease analysis, X. campestris pv. citrumelo does not appear to be a collection of diverse pathovars. Other pathovars of X. campestris may have been identified as X. campestris pv. citrumelo based on pathogenicity; if so, however, these pathovars all have similar genetic backgrounds.

Restriction endonuclease analysis of infrequently occurring recognition sites and conventional RFLPs generated by southern blotting and probing, both generate polymorphisms based on sequence changes, deletions, insertions, and genomic rearrangements. Similarity values generated by these two techniques might be expected to be similar. Similarities for comparisons between X. campestris pv. citri group A are slightly higher for restriction endonuclease analysis than for either RFLP analyses. One reason for this was that the author only had access to strains of X. campestris pv. citri group A from Florida, while strains from many geographical regions were available for the RFLP analyses. The slightly different restriction pattern of strain T1 of X. campestris pv. citri group A is probably because it was isolated from a single citrus tree in Gainesville, FL, and probably had a different origin than

the other strains. All other strains of X. campestris pv. citri group A were isolated from citrus groves and dooryard trees south of Tampa, Florida. In contrast, similarity values for comparisons between strains of X. campestris pv. citri group B were lower for restriction endonuclease analysis than for either RFLP analyses. Restriction endonuclease analysis of infrequently occurring recognition sites as performed here can detect genomic differences at 12 to 15 sites with a single enzyme. Interlocus variation can best be overcome by sampling many areas within the genome. The great power of this type of restriction endonuclease analysis is the ability to detect large scale genomic rearrangements. At present, however, determining whether comigrating bands of diverse strains represent analogous DNA is difficult (Cooksey and Graham, 1989). For closely related strains, restriction endonuclease analysis of infrequently occurring recognition sites is a relatively simple technique that gives results similar to conventional RFLP analysis.

In general, the trends noted for characterization techniques used for within group comparisons were valid for between group comparisons. Comparisons between stains of X. campestris pv. citrumelo and either strains of X. campestris pv. citri group A or B yielded higher similarities with DNA reassociation than the corresponding RFLP analyses. However, when the X. campestris pv. citri groups A and B were

compared, Hartung and Civerolo (1989) RFLP comparisons were very similar to the DNA reassociation values, ca. 60%. It is possible that the latter values were similar because both X. campestris pv. citri groups are relatively homogeneous, whereas comparisons involving the heterogenous strains of X. campestris pv. citrumelo yielded lower RFLP values. As in group comparisons, Gabriel et al. (1989) RFLP comparisons between groups tended to be lower than any other comparison.

Isozyme analysis of between strain similarities indicates a correspondence of isozyme and DNA reassociation values. Similarities between the values generated by these techniques has been noted previously (Gilmour et al., 1987). It is also of interest to note the isozyme variation within the strains of X. campestris pv. citrumelo is similar to the difference between the X. campestris pv. citri group A and strains of X. campestris pv. citrumelo. The large number of isomorphs observed within the X. campestris pv. citrumelo group led to a high degree of variability within X. campestris pv. citrumelo although sequence divergence is not great as measured by DNA reassociation.

The presence of published characterizations of X. campestris pv. citrumelo and X. campestris pv. citri offered an excellent opportunity to compare several bacterial characterization techniques. One of the difficulties in making these comparisons was that the published accounts dealt with different numbers of comparisons and, at times,

different strains. All investigators placed strains in the same groups (three groups of X. campestris pv. citrumelo and X. campestris pv. citri groups A and B), however, lending validity to comparisons between investigations. At the very least, perhaps the above discussion will encourage further investigations into the relationships between techniques.

The techniques used in this study, DNA reassociation and restriction endonuclease analysis of infrequently occurring recognition sites, complement each other well in the characterization of bacterial strains. The total genomic distance between bacterial strains or groups of strains may be estimated by DNA reassociation, while restriction endonuclease analysis indicates the relatedness between similar strains. Several techniques are ultimately necessary in the characterization of any bacterial group. Relationships derived by the use of just one technique could be misleading, yet upon consideration of several techniques, each can be put into its proper context.

It has been proposed that Xanthomonas campestris pv. citri be elevated to species status solely on the basis of unique RFLP patterns using the authors probes (Gabriel et al., 1989). Such a proposal was premature since insufficient data was presented (Vauterin et al., 1990a). In addition, DNA reassociation values have been recommended for such a proposal (Wayne et al., 1987). RFLP values can be combined with DNA reassociation studies as well as other

techniques (Vauterin et al., 1990a). The comparison of the data presented here with RFLP data of others indicates that RFLP and DNA reassociation data are not necessarily synonymous.

Although it has been shown here that strains of X. campestris pv. citri group A are only ca. 60% related to the type strain of X. campestris pv. campestris, this is not sufficient for the elevation of the former strains to species status (Wayne et al., 1987). The work presented herein, however, may facilitate the understanding of the relationships among strains of X. campestris pv. citrumelo and X. campestris pv. citri as well as other pathovars of X. campestris. It is hoped that by combining the data presented here with additional data generated through different techniques, a stable classification of these important pathogens may result.

CHAPTER 5
CHARACTERIZATION WITH AN *hrp* GENE CLUSTER

Xanthomonas campestris pv. *citrumelo* causes Citrus bacterial spot primarily on Swingle citrumelo and grapefruit varieties. Three aggressiveness groups have been identified among strains of *X. campestris* pv. *citrumelo* (Graham and Gottwald, 1990). The least aggressive strains cause small (< 1 mm), slightly raised lesions with little or no watersoaking or chlorosis (Graham et al., 1990a; Graham and Gottwald, 1990). Both weakly and moderately aggressive strains are usually associated with injury to the plant (Gottwald and Graham, 1990; Graham and Gottwald, 1990).

One possible explanation for the limited aggressiveness of many strains of *X. campestris* pv. *citrumelo* is that these strains are opportunistic. Opportunistic xanthomonads have been isolated from decayed vegetables (Liao and Wells, 1977) and from tomato and pepper transplants (Gitaitis et al., 1977). These strains, like many strains of *X. campestris* pv. *citrumelo*, are limited in pathogenicity and are pectate positive (Gitaitis et al., 1977).

Opportunistic xanthomonads differ from phytopathogenic xanthomonads by lacking an intact *hrp* gene cluster. The *hrp* gene cluster is required by plant pathogens to produce symptoms on susceptible hosts and a hypersensitive reaction on nonhosts (U. Bonas et al., in press; Boucher et al.,

1987; Lindgren et al., 1986). An hrp gene cluster has been discovered in Pseudomonas syringae pv. phaseolicola (Lindgren et al., 1986), Pseudomonas solanacearum (Boucher et al., 1987) and Xanthomonas campestris pv. vesicatoria (U. Bonas et al., in press). Genomic DNA of opportunistic xanthomonads hybridized weakly or not at all to the hrp gene cluster from X. campestris pv. vesicatoria, whereas genomic DNA from 33 pathovars of X. campestris did hybridize, but fragments were polymorphic (Stall and Minsavage, 1991). Strains of X. campestris from citrus, which lacked pathogenicity on that host, also lacked an hrp gene cluster homologous to the hrp gene cluster from X. campestris pv. vesicatoria (J. Graham unpublished). If the less aggressive strains of X. campestris pv. citrumelo are opportunistic on citrus, the hrp region might be absent from these strains. This hypothesis might help explain the origin and the pathogenicity of many strains of X. campestris pv. citrumelo.

Alternatively, the less aggressive strains of X. campestris pv. citrumelo may be representatives of other X. campestris pathovars that have incidentally been isolated from citrus (Gabriel et al., 1989; Graham et al., 1990c). These strains are diverse and some can cause symptoms on noncitrus hosts (Gabriel et al., 1989; Graham et al., 1990c). In this case, the hrp gene cluster should be present but display polymorphisms between pathovars (Stall and Minsavage, 1991).

Plasmid profiles or plasmid sequence analyses have been used to distinguish pathovars of X. campestris (Lazo and Gabriel, 1987). If strains X. campestris pv. citrumelo represent several diverse pathovars of X. campestris, these strains may also possess diverse plasmid profiles. In addition, plasmid profiles of strains of X. campestris pv. citrumelo may be similar to known plasmid profiles of strains of X. campestris pv. citri groups A and B which cause similar symptoms on related hosts (Civerolo, 1985a).

To test these hypotheses, genomic DNA of weakly aggressive and moderately aggressive strains of X. campestris pv. citrumelo were probed with the hrp gene cluster from X. campestris pv. vesicatoria. Highly aggressive strains of X. campestris pv. citrumelo and strains of X. campestris pv. citri group A were probed for comparison purposes. Plasmid profiles of these strains were studied to determine whether plasmid profiles could be used to distinguish between the less aggressive strains of X. campestris pv. citrumelo as might be expected if these strains belonged to different pathovars of X. campestris.

Materials and Methods

Culture conditions. The taxonomic designations and sources of all strains are listed in Table A-1. Strains were cultured on nutrient agar (BBL Microbiology Systems, Cockeysville, MD) or Lima bean agar (Difco, Detroit, MI) and single colonies were selected for use. Cells were grown in

nutrient broth (BBL Microbiology Systems) overnight (20-24 hrs) or, for X. campestris pv. citri group B, in a sucrose based medium (Canteros de Echenique et al., 1985) for 30-36 hrs. Long term storage was achieved in glycerol/nutrient broth (85/15%, v/v) at -70 C.

Hybridization analysis. Genomic DNA was isolated by the procedure of Boucher et al. (1987) and restricted with the appropriate restriction endonuclease for 2 hr at 37 C. Samples were then treated with RNase A (Sigma, Ribonuclease A, type II-A) for 0.5 hr and electrophoresed in 0.5% agarose gel containing TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) at 2V/cm of gel. The gel was then denatured in 0.4 N NaOH and 0.6 M NaCl for 0.5 hr and neutralized for 0.5 hr in 0.5 M Tris, 1.5 M NaCl. The denatured DNA was then transferred by the procedure of Southern (1975) to a nylon membrane (Gene Screen Plus, DuPont, Boston, MA). Hybridizations were performed at 68 C (without formamide) for 18 to 24 hrs and washed at 68 C with 0.1 X SSC, 0.1% w/v sodium dodecyl sulfate (SDS). Probes were labelled by the random primed method and detected by the use of the Genius Nonradioactive DNA Labelling and Detection kit (Boerhinger Mannheim, Indianapolis, IN) according to the manufacturers instructions. The probe was the hrp gene cluster 993 of X. campestris pv. vesicatoria (Stall and Minsavage, 1991) as an insert of pLAFR-3.

Plasmid analysis. Plasmid profiles were determined by a modification of a previously published procedure (Kado and Liu, 1981). Bacterial cells (5 x 10^8 cfu/ml) were pelleted in a 1.5 ml centrifuge tube in a microcentrifuge, washed in sterile distilled water and resuspended in 50 µl TAE buffer. The bacterial cells were then lysed for 15 min at 30 C in 400 µl of 50 mM Tris, 0.57 M sodium chloride, 0.04 M sodium hydroxide and 3% w/v SDS. The lysate was extracted with 2 volumes of phenol (Fisher): chloroform: isoamyl alcohol (25:24:1). The supernatant was electrophoresised in 0.5% agarose gels in tris-acetate buffer at 5 V/cm. Gels were stained in 0.5 mg ethidium bromide per liter. Plasmids of strain SW2 of Erwinia stewartii were used as molecular markers (Coplin et al., 1981). Molecular weights were determined by regressing the $\log_{10}$ of the molecular weight versus the $\log_{10}$ of the relative mobility of the plasmids as determined from Polaroid type 55 photographs.

Results

Hybridization analysis. The hrp gene cluster hybridized to genomic DNA of all strains of X. campestris pv. citrumelo regardless of aggressiveness type (Fig. 5-1). Genomic DNA of the two strains of X. campestris pv. citri group A also hybridized with the hrp gene cluster. The hrp gene cluster restriction patterns generated with Eco RI were similar for strains of X. campestris pv. citrumelo of

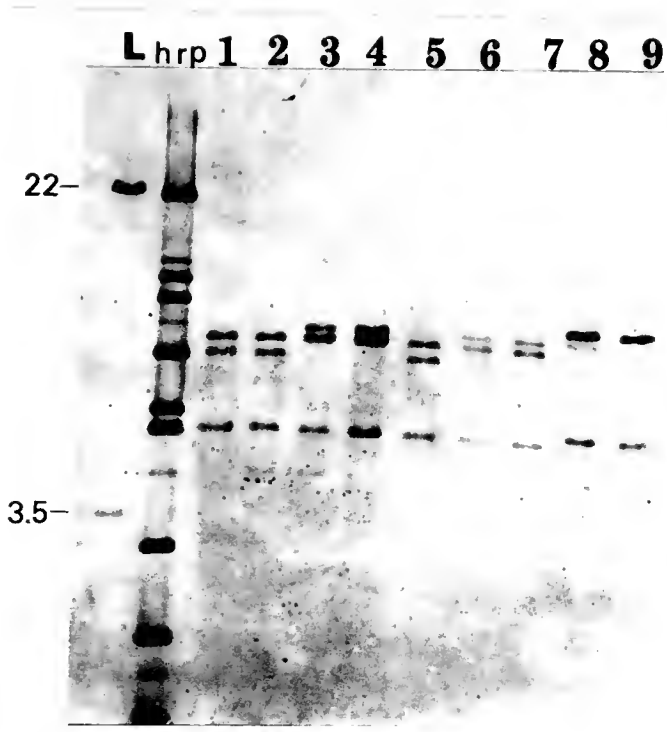


Figure 5-1. Hybridization of the hrp gene cluster from Xanthomonas campestris pv. vesicatoria to genomic DNA of strains of X. campestris pv. citrumelo and X. campestris pv. citri group A. Lane L, phage λ restricted with Hind III and Eco RI; lane hrp, hrp gene cluster 993 restricted with Eco RI. Lanes 1 to 9 represent genomic DNA restricted with Eco RI. Lanes 1 to 7 are strains of X. c. pv. citrumelo. Lane 1 is strain 540 isolated from Clausena wampi; lanes 2 and 3 are weakly aggressive strains F306 and F59; lanes 4 and 5 are moderately aggressive strains F254 and F6; lanes 6 and 7 are highly aggressive strains F54 and F1. Lanes 8 and 9 are strains 9771 and 3213 of X. c. citri group A. Molecular sizes are given in kilobases.

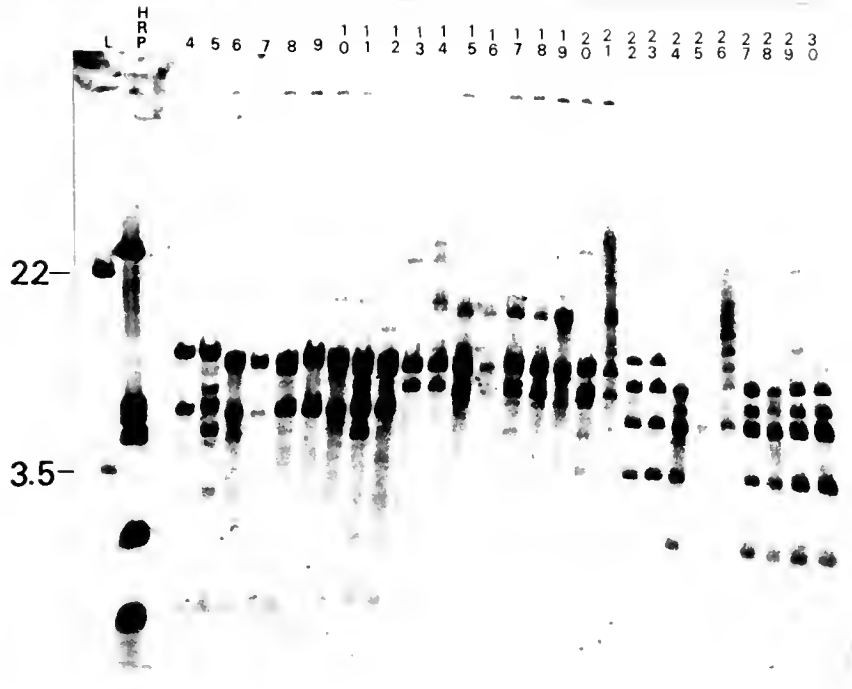


Figure 5-2. Hybridization of the hrp gene cluster from Xanthomonas campestris pv. vesicatoria to genomic DNA of strains of X. campestris pv. citrumelo and X. campestris pv. citri group A. Lane L, phage λ restricted with Hind III and Eco RI; lane HRP, hrp gene cluster 993 restricted with Eco RI; lanes 4 to 12 represent genomic DNA restricted with Eco RI. Lanes 4 and 5, are strains of X. c. pv. citri 3213 and 9771; Lanes 6 to 12 are strains of X. c. pv. citrumelo. Lanes 6 and 7 are highly aggressive strains F1 and F361; lanes 8 and 9 are moderately aggressive strains F6 and F254; lanes 10 and 11 are weakly aggressive strains F100 and F306; lane 12 is strain 534 isolated from Clausena wampi. Lanes 13 to 21 are the same as 4 to 12, except restricted with Bam HI. Lanes 22 to 30 are the same as 4 to 12, except restricted with Eco RV. Molecular sizes are given in kilobases.

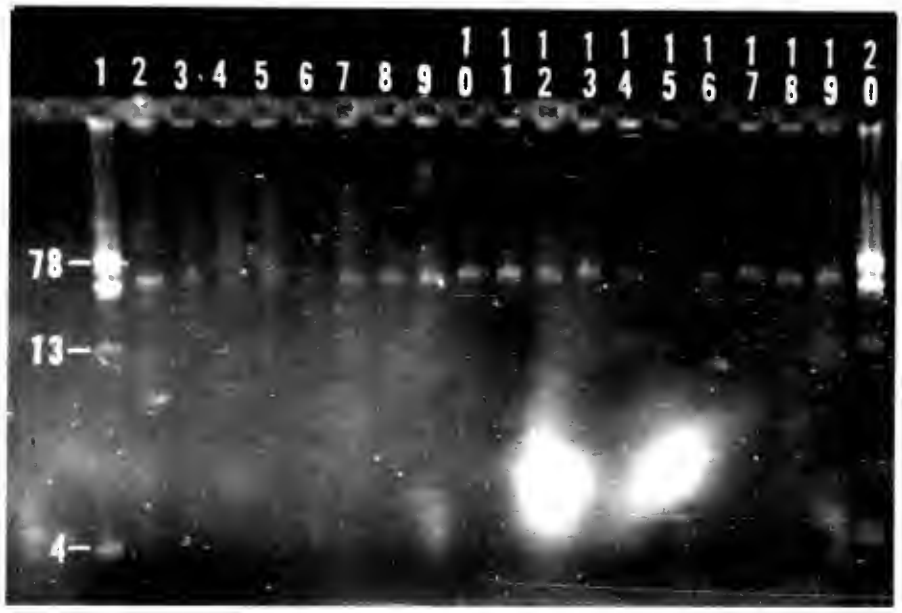


Figure 5-3. Plasmid profiles of Xanthomonas campestris pv. citrumelo from Florida. Lanes 1 and 20 are Erwinia stewartii plasmids as molecular size markers (labelled in kilobases). Lanes: 2, F1; 3, F274; 4, F54; 5, F361; 6, F5; 7, 84-3166; 8, F228; 9, F6; 10, F254; 11, F299; 12, F311; 13, F348; 14, 89-3274; 15, F397; 16, F100; 17, F306; 18, F86; 19, F94.

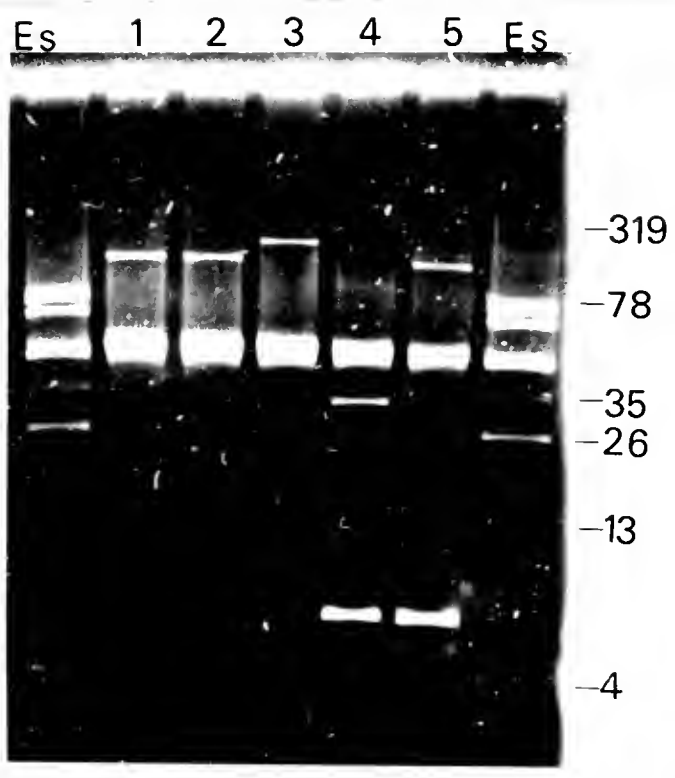


Figure 5-4. Plasmid profiles of Xanthomonas campestris pv. citri group A from Florida. Lanes Es are Erwinia stewartii plasmids as molecular size markers (labelled in kilobases). Lanes: 1, T1; 2, 9760; 3, 3340; 4, 3213; 5, 9771.

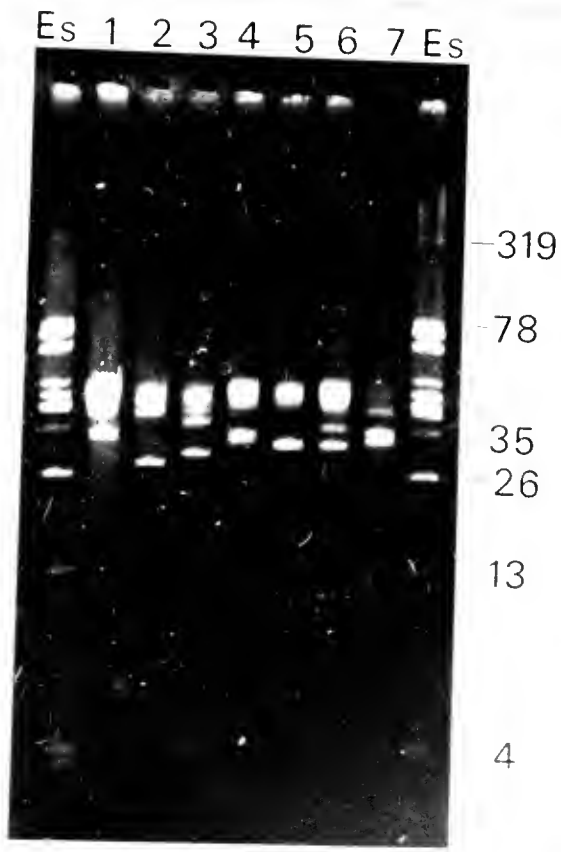


Figure 5-5. Plasmid profiles of strains of Xanthomonas campestris pv. citri group B from South America. Lanes Es are plasmids from Erwinia stewartii used as molecular size markers (labelled in kilobases). Lanes: 1, B84; 2, B93; 3, B148; 4, B80; 5, B64; 6, B69; 7, B94.

Table 5-1. Size classes of plasmids found in strains of Xanthomonas campestris pv. citri group A from Florida.

	Strain				
	T1	9760	3340	3218	9771
1.				190.6[a,b]	
2.	148.4	148.4			131.8
3.				31.0	
4.				11.8	11.6
5.				6.2	6.1
6.				5.2	5.2

[a]Size in kilobases.
[b]Plasmids sized by Erwinia stewartii size standards (Coplin et al., 1981).

Table 5-2. Plasmid size classes of strains of *Xanthomonas campestris* pv. *citri* group B from South America.

	Strains						
	B84	B93	B148	B80	B64	B69	B94
1.		43.5[a,b]	45.8				41.3
2.	37.5	37.5	35.7	32.5		35.7	34.2
3.	29.8			31.2	29.8	29.8	31.2
4.	28.6		27.4				
5.		25.1					

[a]Size in kilobases.
[b]Plasmids sized by *Erwinia stewartii* size standards (Coplin *et al.*, 1981).

different aggressiveness types and the few polymorphisms that were observed did not correspond to aggressiveness type (Fig. 5-1). Restriction patterns of genomic DNA of strains of X. campestris pv. citrumelo and X. campestris pv. citri group A were different when restricted with Eco RI and probed with the hrp gene cluster (Fig. 5-1).

In order to investigate these relationships further, a hybridization analysis was conducted with two additional restriction endonucleases (Fig. 5-2). Restriction patterns for strains of X. campestris pv. citrumelo probed with the hrp gene cluster were similar for all three restriction endonucleases, Eco RI, Bam HI, and Eco RV. Eco RV and Bam HI digests of genomic DNA of X. campestris pv. citrumelo and X. campestris pv. citri group A produced several comigrating bands when probed with the hrp gene cluster.

Plasmid analysis. No plasmids were observed for any strain of X. campestris pv. citrumelo (Fig. 5-3). Strains of X. campestris pv. citri group A from Florida display at least six plasmid size classes (Fig. 5-4, Table 5-1). Strains T1, 9760 and 9771 contain a plasmid ranging in size from 131.8 to 148.4 kb, while strain 3340 contains a 190.6 kb plasmid. In contrast, the largest plasmid in strain 3213 is 31 kb. Strains 3213 and 9771 also contain several smaller plasmids (Fig. 5-4 and Table 5-1).

The seven strains of X. campestris pv. citri group B represent seven different plasmid profiles (Fig. 5-5).

Plasmids ranged in size from 25.1 to 45.8 kb (Table 5-2). Some 13 different plasmids are represented on the basis of size.

Discussion

All strains of X. campestris pv. citrumelo studied here have an hrp region as demonstrated by hybridization of the X. campestris pv. vesicatoria hrp gene cluster to genomic DNA of the above strains. Strains of opportunistic xanthomonads, exhibiting limited pathogenicity (Gitaitas et al., 1977), were observed to lack an hrp region homologous with the hrp gene cluster from X. campestris pv. vesicatoria (Stall and Minsavage, 1991). Although some strains of X. campestris pv. citrumelo are only weakly to moderately aggressive on citrus, the presence of an hrp gene cluster is not consistent with an opportunistic etiology for these pathogens on citrus. It is possible that the hrp gene cluster in strains of X. campestris pv. citrumelo, while shown here to be present, is not expressed. However, a hypersensitive reaction is exhibited upon inoculation of strains of X. campestris pv. citrumelo into tobacco (Stall, unpublished data).

Alternatively, the limited pathogenicity of some strains of X. campestris pv. citrumelo and the presence of an hrp gene cluster could be explained if these strains belonged to other pathovars of X. campestris (Gabriel et al., 1989; Graham et al., 1990c). The hrp gene cluster

exhibited a diverse array of polymorphisms with 33 different pathovars of X. campestris (Stall and Minsavage, 1991); however, the strains studied here had similar restriction patterns. It does not appear that unrelated pathovars were coincidentally associated with wounds on citrus. Some strains of X. campestris pv. citrumelo appear to have a host range which includes noncitrus hosts (Gabriel et al., 1989; Graham et al., 1990c). However, this may be evidence for a single pathovar with a large host range.

Plasmid profiles of the strains of X. campestris pv. citrumelo did not help resolve the pathovar status of these strains. No plasmids were observed for these strains, although plasmids have been reported elsewhere for some of these strains (Gabriel et al., 1989). Although care was taken to only utilize single colonies from plates streaked directly from ice cultures, some plasmids may have been lost. Plasmids are not always stable and may be easily lost (e.g., Minsavage et al., 1990).

The plasmid size classes and profiles found here do not correspond completely with data reported earlier for X. campestris pv. citri groups A and B (Civerolo, 1985a). Four out of five strains of X. campestris pv. citri group A contained a plasmid of 131.8 to 190.6 kb. The largest plasmid of a previous report was 82.1 kb (Civerolo, 1985a). The strains of X. campestris pv. citri group A examined here

all came from Florida, whereas the previous report predated the presence X. campestris pv. citri group A in Florida.

Plasmid profiles of strains of X. campestris pv. citri group B were diverse and individual plasmids ranged in size from 25.1 to 45.8 kb. Strains B64 and B69 were analyzed for plasmids previously (Civerolo, 1985a). The results presented here are similar, although in strain B69, a 48 kb plasmid is missing and has been replaced with a 35 kb plasmid.

No single plasmid size class or plasmid profile was associated with either X. campestris pv. citri group A or B and several plasmid profiles were observed within each group of X. campestris pv. citri. Thus, plasmid profiles do not appear useful in identifying either of these two groups. This diversity in plasmid profiles limits the diagnostic values of this method with these strains.

Some of the plasmid profiles reported here differ from previous reports (Civerolo, 1985a; Gabriel et al., 1989). This may be due to changes which occur in routine culture and handling of strains. In any case, this type of phenomenon might also limit the usefulness of plasmids in discriminating these groups.

The sudden appearance of a diverse array of strains of X. campestris pv. citrumelo exhibiting a range of aggressiveness on citrus hosts remains unexplained. Since strains of X. campestris pv. citrumelo have an hrp gene

cluster, these strains are probably true pathogens, although weakly aggressive. It is possible that some strains of X. campestris pv. citrumelo are more aggressive on noncitrus hosts. However, based on the similar restriction patterns for the hrp gene clusters of these strains, X. campestris pv. citrumelo probably does not consist of several pathovars of X. campestris. Strains of X. campestris pv. citrumelo may comprise a group of related strains with a broad host range.

CHAPTER 6
CARBOHYDRATE UTILIZATION

Strains of X. campestris pv. citrumelo which cause Citrus bacterial spot in Florida have been reported to be diverse pathogenically and genetically. Three different aggressiveness groups of X. campestris pv. citrumelo have been identified by detached and attached leaf assays (Graham and Gottwald, 1990). Genetic variation between strains of X. campestris pv. citrumelo have been observed with RFLP analyses (Gabriel et al., 1989; Graham et al., 1990c; Hartung and Civerolo, 1989). Aggressiveness types have been distinguished by RFLP analyses (Gabriel et al., 1989; Hartung and Civerolo, 1989).

Strains of X. campestris pv. citri group A, causal agent of Asiatic citrus canker, have also been found in Florida. These strains are distinguishable from X. campestris pv. citrumelo pathogenically and by RFLP analysis (Gabriel et al., 1989; Graham and Gottwald, 1990; Hartung and Civerolo, 1989). Strains of X. campestris pv. citri group A, however, were reported to be much more homogeneous genetically (Gabriel et al., 1989; Hartung and Civerolo, 1989).

Considering the pathogenic differences in these strains, it is not surprising that genetic differences are also observed. It is not known, however, what other phenotypic traits vary among these strains. Carbohydrate utilization varies between groups of X. campestris pv. citri (Goto et al., 1980a). Carbohydrate utilization patterns may also vary among strains of X. campestris pv. citrumelo aggressiveness types and between strains of X. campestris pv. citrumelo and X. campestris pv. citri group A. If X. campestris pv. citrumelo represents an assemblage of diverse strains with the ability to cause symptoms on citrus, perhaps carbohydrate utilization patterns are also diverse.

Carbohydrate utilization patterns among these strains were compared using Biolog microtiter plates. Each plate contains 95 carbon sources, allowing catabolic fingerprints of bacterial strains to be rapidly compared. Biolog library generation software was also used to compare groups of strains.

Materials and Methods

Bacterial strains, their taxonomic designations, and sources are listed in Table A-1.

Library generation comparisons. Methods used were those recommended by the manufacturer of Biolog GN plates (Biolog, Inc., Hayward, CA). Culture media of trypticase soy broth agar (BBL Microbiology Systems, Cockeysville, MD) were inoculated with single colonies and incubated for 24

hrs at 30 C. Cells were removed from the agar by rolling a sterile cotton swab across the surface and resuspending the cells in 0.85% sterile sodium chloride until a suspension of 5×10^8 cells/ml was obtained (0.3 OD at 600 nm). Each well of the microtiter plate was filled with 150 μl of the bacterial suspension. Microtiter plates were incubated at 27 C and absorbance at 590 nm read on a microtiter plate reader (Biolog) at 4 and 24 hrs.

Using the Biolog Library Generation System, sublibraries were made of weakly, moderately and highly aggressive strains of X. campestris pv. citrumelo. Sublibaries were made of two additional groups which included recently isolated strains that had not been characterized as to aggressiveness. These latter strains were separated into a pectate negative and a pectate positive group and sublibraries were made of each. All strains were assessed for their similarity to those strains initially assigned to the same sublibrary as well as strains in other sublibraries.

Similarity comparisons. When Biolog plates were inoculated as above, strains of X. campestris pv. citri group A caused very few positive reactions among the 95 carbon sources. Therefore, a different procedure was followed to compare strains of X. campestris pv. citri group A with other strains. Saline (0.85% sodium chloride) amended with 0.05% yeast extract (w/v) (Difco, Detroit, MI)

allowed both positive and negative reactions to occur on Biolog plates inoculated with strains of X. campestris pv. citri group A, and did not affect reactions with strains of X. campestris pv. citrumelo. In this procedure, cells were grown in nutrient broth, harvested by centrifugation, and washed with sterile saline before the O.D. was adjusted as above. Wells were inoculated as above. Wells were counted positive when O.D. was above 0.20 at 570 nm (Easy Reader EAR 400 FW, Austria). Similarities were calculated by noting the percentage of similar reactions between plates.

Results

Library generation comparisons. Few positive reactions were recorded at the 4 hr reading, therefore, only 24 hr readings are considered here.

The carbohydrate utilization pattern of each strain was compared to every other strain. Similarity values were generated based on how each strain compared to other strains assigned to that sublibrary. These similarity values were averaged for strains initially assigned to a sublibrary (Table 6-1). The highest similarity values for each sublibrary occur when compared to strains in its own sublibrary. That is, on average, the strains initially assigned to a sublibrary were most similar to other strains assigned to that sublibrary. None of the values presented in Table 6-1 exceed 0.676 because for each group of strains assigned to a sublibrary, some strains were more similar to

Table 6-1. Strains of Xanthomonas campestris pv. citrumelo were assigned to one of five sublibraries and the similarities of each strain to its assigned sublibrary as well as to each other sublibrary is averaged for strains in each sublibrary.

	HAP-[a]	MAP+	WAP+	RIP+	RIP-
HAP-(6)[b]	0.482	0.342	0.093	0.028	0.007
MAP+(9)	0.015	0.610	0.091	0.089	0.015
WAP+(5)	0.145	0.186	0.586	0.002	0.004
RIP+(4)	0.002	0.180	0.065	0.616	0.006
RIP-(6)	0.033	0.203	0.004	0.031	0.676

[a]HAP-=Highly aggressive strain, pectate negative; MAP+=moderately aggressive strain, pectate postive; WAP+=weakly aggressive strain, pectate positive. RIP+=recent isolate whose aggressiveness was uncharacterized, pectate positive. RIP-= recent isolate whose aggressiveness was uncharacterized, pectate negative. Homologous comparisons underlined.
[b]Number of strains in sublibrary.

Table 6-2. Strains of Xanthomonas campestris pv. citrumelo were assigned to one of five sublibraries; each strain is placed under the sublibrary it is most similar to in descending order.

A HAP-[a]		B MAP+		C WAP+		D RIP+		E RIP-	
F1	0.913	F299	0.873	F94	0.873	5429	0.899	4929-3	0.884
F274	0.799	F6	0.868	F86	0.835	5573-1	0.894	5532-2	0.884
F306[C]	0.724[b]	F228	0.776	F100	0.695	F254[B]	0.737	4929-1	0.868
F361	0.655	F348	0.764	F59	0.525	5560-1	0.590	5439-1	0.814
F5	0.475	F54[A]	0.751	3166[A]	0.523	F361[A]	0.140	4929-2	0.418
4929-2[E]	0.158	F311	0.696	6261	0.291	5532-1[E]	0.103	5532-1	0.187

[a]HAP-=Highly aggressive strains, pectate negative; MAP+=moderately aggressive strains, pectate positive; WAP+=weakly aggressive strain, pectate positive; RIP+=recent isolate, aggressiveness uncharacterized, pectate positive; RIP-=recent isolate, aggressiveness uncharacterized, pectate negative.

[b] Underlined entries are strains which were more similar to another sublibrary than to the one to which they were originally assigned. Letter indicates originally assigned sublibrary.

Table 6-3. Similarity matrix generated by carbohydrate utilization patterns of strains of Xanthomonas campestris pv. citrumelo, X. campestris pv. citri group A, X. campestris pv. campestris and X. maltophilia.

	Xanthomonas campestris pv.					X. maltophilia
	citrumelo			citri A	campestris	
	F1	F6	F100	9771	33913	1540
F1	--	93[a]	90	81	82	77
F6	--	--	98	88	85	76
F100	--	--	--	90	85	76
9771	--	--	--	--	82	75
33913	--	--	--	--	--	67
1540	--	--	--	--	--	--

[a]Values in percent.

strains assigned to different sublibraries. Those strains which were most similar to a particular sublibrary and the accompanying similarity values of that strain for that sublibrary are presented in Table 6-2 in descending order of similarity. Strains initially assigned to a sublibrary were occasionally more similar to strains assigned to a different sublibrary. For example, weakly aggressive strain F306 of X. campestris pv. citrumelo had a similarity value of 0.724 to strains assigned to the highly aggressive sublibrary. Highly aggressive strain F54 had a similarity value of 0.751 to the moderately aggressive sublibrary. All six strains listed under the moderately aggressive sublibrary are more than 0.5 similar to that library. This occurs in no other sublibrary.

Similarity comparisons. Since strains of X. campestris pv. citri group A caused very few positive reactions on microtiter plates inoculated without 0.05% yeast extract, these strains were tested for auxotrophy on minimal medium. However, all strains of X. campestris tested grew on minimal media.

A similarity matrix including representatives of each X. campestris pv. citrumelo aggressiveness group, strain 9771 of X. campestris pv. citri group A, the type strain 33913 of X. campestris pv. campestris and a strain of X. maltophilia is presented in Table 6-3. Strains of X. campestris pv. citrumelo and X. campestris pv. citri group A

average 90% related to each other. Strain 33913 of X. campestris pv. campestris averaged 83% related to strains of X. campestris pv. citrumelo and X. campestris pv. citri group A. Similarities of strains of X. maltophilia to strains of X. campestris pv. citrumelo and X. campestris pv. citri group A are lower, averaging 76%. Strain 33913 of X. campestris pv. campestris was only 67% related to X. maltophilia.

Discussion

The aggressiveness of strains of X. campestris pv. citrumelo is not necessarily correlated with carbohydrate utilization patterns. The highly aggressive group of strains of X. campestris pv. citrumelo are relatively homogeneous based on RFLP analyses; however, two highly aggressive strains had carbohydrate patterns which were more similar to weakly or moderately aggressiveness groups. A clonal group of bacteria would be expected to have similar pathogenic and catabolic capabilities. However, in some cases, pathogenic ability does not appear to be correlated with carbohydrate utilization abilities.

The lack of pectate degrading ability of strains of X. campestris pv. citrumelo corresponds to highly aggressive strains (Stall, unpublished data). Recently isolated pectate negative strains of X. campestris pv. citrumelo did not fit in well to the other three aggressiveness groups. In fact, most of these strains fit best within their own

sublibrary. Recently isolated pectate positive strains of X. campestris pv. citrumelo also fit in best to their own sublibrary, although a moderately aggressive strain also was similar to this group. Recently isolated pectate positive and pectate negative strains were ranked moderately aggressive in a detached leaf assay (E. L. Civerolo, personal communication). The recently isolated pectate negative strains have been separated from previously isolated pectate negative strains both in aggressiveness and carbohydrate utilization patterns. Although the recently isolated pectate positive strains are similar to previously isolated moderately aggressive strains, this new group appears different in carbohydrate utilization patterns.

All strains of X. campestris ranged from 81 to 98% similar to one another by carbohydrate utilization patterns regardless of pathovar status. Although an RFLP similarity value of 0.63 exists, for example, between F1 and F100 (Graham et al., 1990c), these strains had similar carbohydrate utilization patterns. A similar situation exists for Pseudomonas syringae pv. tomato where carbohydrate utilization patterns are similar, although RFLP analyses indicate diversity (Denny, 1988; Denny et al., 1988). Isozyme and RFLP analyses have both indicated differences between X. campestris pv. citrumelo and X. campestris pv. citri group A; however, carbohydrate utilization patterns were similar between representatives of

these pathovars. A recent survey of 266 strains of the genus Xanthomonas compared 295 phenotypic features of these strains (Van der Mooter and Swings, 1990). These authors found that the 189 strains of X. campestris were 87.2% similar. These results are similar to the data presented here. These similarities stand in contrast to studies using DNA reassociation experiments which indicate large genetic diversity among pathovars of X. campestris (Vauterin et al., 1990a). The lowest similarity values recorded here were of X. maltophilia compared to any strain of X. campestris. However, these values were not as low as previously recorded for this comparison (Van der Mooter and Swings, 1990).

Carbohydrate utilization patterns have been used to show differences within aggressiveness groups of X. campestris pv. citrumelo, yet overall differences among all strains of X. campestris were small. The difference between the similarities observed here and those obtained elsewhere with different techniques (Gabriel et al., 1988; Gabriel et al., 1989; Graham et al., 1990c; Hartung and Civerolo, 1989) emphasize the importance of considering more than one technique for the characterization of bacterial strains. Phenotypic features among strains of X. campestris are similar, perhaps limiting the usefulness of carbohydrate utilization patterns within this taxon.

CHAPTER 7
DISCUSSION

Citrus canker diseases and Citrus bacterial spot are unusual diseases in that they are all caused by taxonomically related, yet distinctly different groups of Xanthomonas campestris. The genomic and pathological relationships of these strains and the diseases they cause form the basis of this work. Questions concerning the extent of variation within some of these groups of strains also led to the experimentation described herein.

One of the unique observations of Citrus bacterial spot was that there was wide variation in the aggressiveness of strains (Graham and Gottwald, 1988). These aggressiveness groups of X. campestris pv. citrumelo can be separated by bacterial populations produced on and in leaves and lesion expansion based on inoculations of citrus varieties in the field as reported here. Weakly aggressive strains produced lesions < 1 mm in diameter on Duncan grapefruit and produced no internal populations after 50 days.

Graham et al. (1990c) suggested that only highly aggressive strains of X. campestris pv. citrumelo should be included in that pathovar because of the highly aggressive nature and the relative homogeneity of these strains, both

pathogenically and genetically. The highly aggressive strains caused relatively large, watersoaked lesions and were distinguished pathogenically from moderately and weakly aggressive strains by population development in and on citrus leaves, the extent of lesion expansion on Swingle citrumelo, and by a host interaction on Swingle citrumelo and Duncan grapefruit (Chapter 3; Graham et al., 1990a). The highly aggressive strains were also pectate negative in contrast to the pectate positive character of weakly and moderately aggressive strains (Stall, unpublished data). This latter trait might be used to differentiate X. campestris pv. citrumelo (sensu stricto) from other strains causing Citrus bacterial spot.

If only highly aggressive strains are included in X. campestris pv. citrumelo, the less aggressive strains of this group are left without taxonomic affinity. It is possible these strains are more aggressive on noncitrus hosts, but ascertaining this, and what hosts may be involved has not been accomplished. Under the present pathovar system, a strain of X. campestris not associated with a host or a strain of X. campestris that is associated with an unreported disease is difficult to identify. It is not surprising that many pathovars have been named based on a new host report. If the weakly and moderately aggressive strains are given separate pathovar designations there needs to be justification based on alternate hosts or symptom production.

Progress has been made recently in separating X. campestris into at least six DNA homology groups (Vauterin et al., 1990a). It may be possible to assign X. campestris into several species based on this approach (Vauterin et al., 1990a), but species should also correspond to groups separable by a sufficient number of physiological tests. Although placing strains into a particular pathovar of a species would still be difficult, each species would include related pathovars.

Strains of X. campestris pv. citrumelo, X. campestris pv. citri groups A and B are genomically distinct from one another by DNA reassociation. However, the diseases, Citrus bacterial spot, Asiatic citrus canker and Cancrosis B have similar symptoms on related hosts. The similar symptoms produced by these strains might be due to the same genes for pathogenicity, which occupy relatively little genetic information, on diverse genetic backgrounds. In contrast, it may be possible that the genes which code for pathogenicity and symptom production are different, involve a large proportion of the genome and yet produce similar phenotypes. An analogous situation may account for similar carbohydrate utilization patterns as observed in this study. It is possible that the 95 carbon sources studied here require relatively little genetic information, so that genetic differences between these strains exist in parts of the genome not related to carbohydrate utilization. On the

other hand, the same carbohydrate utilization patterns could be the result of different genes that form isozymes. This may be common in X. campestris because when 295 phenotypic traits were studied, 189 strains of X. campestris were 87.2% similar (Van den Mooter and Swings, 1990). This result contrasts with the fact that this species encompasses large genetic differences based on DNA reassociation (Vauterin et al., 1990a).

Strains of X. campestris pv. citrumelo are genetically related, by DNA reassociation data from this study and RFLP data (Graham et al., 1990c), to a group of strains from noncitrus hosts which cause lesions on citrus. These strains consist of ornamental pathogens from Florida and X. campestris pv. alfalfae. Strains could probably be isolated from other plants which produce a phenotype on citrus similar to the less aggressive strains of X. campestris pv. citrumelo. Conversely, some of the less aggressive strains of X. campestris pv. citrumelo may also cause disease on noncitrus hosts. These alternate hosts may have served as a reservoir for strains pathogenic to citrus which were ultimately identified as X. campestris pv. citrumelo. In this study, a weakly aggressive strain of X. campestris pv. citrumelo has been detected on citrus leaves in the absence of internal populations. This observation may indicate that these strains are capable of epiphytic survival on citrus. Such epiphytic capabilities would account for how these

strains survive and spread. These strains may be representative of a group of genetically similar strains which cause symptoms on a variety of hosts.

The primary method of genomic analysis chosen for this study of xanthomonads from citrus was DNA reassociation. Other techniques such as RFLP and isozyme analysis indicate wide diversity within strains of X. campestris pv. citrumelo (Gabriel et al., 1989; Graham et al., 1990c; Hartung and Civerolo, 1989; Kubicek et al., 1989). None of these techniques examine the entire genome, however, as does DNA reassociation. The major finding of this study is that strains of the three aggressiveness groups of X. campestris pv. citrumelo are closely related (ca. 88%). Two practical implications that may be deduced from this finding concern the origin of the strains of X. campestris pv. citrumelo and the techniques used to characterize bacterial strains.

At least three alternative hypotheses explain the origin of the weakly and moderately aggressive strains of X. campestris pv. citrumelo. These strains are often associated with injury to the plant (Gottwald and Graham, 1990; Graham and Gottwald, 1990) and may not be able to induce symptoms without injury. That is, some strains of X. campestris pv. citrumelo may be opportunistically associated with lesions on citrus. Such opportunistic strains, however, should lack an hrp gene cluster (Stall and Minsavage, 1991) which all strains of X. campestris pv.

citrumelo tested here possessed. On this basis, these strains do not appear to be opportunistic.

Alternatively, strains of X. campestris pv. citrumelo may be pathogens normally associated with noncitrus hosts, i.e., these strains may represent several pathovars of X. campestris. The weakly and moderately aggressive strains of X. campestris pv. citrumelo are diverse based on RFLP data (Gabriel et al., 1989; Graham et al., 1990c; Hartung and Civerolo, 1989) as would be expected if X. campestris pv. citrumelo consisted of several pathovars of X. campestris associated with lesions on citrus solely due to their existence on adjacent vegetation. Pathovars of X. campestris not normally associated with citrus can cause symptoms on citrus (Gabriel et al., 1989; Graham et al., 1990c) and these strains are closely related to strains of X. campestris pv. citrumelo based on DNA reassociation experiments reported here. Therefore, strains of X. campestris pv. citrumelo may include strains normally associated with noncitrus hosts and thus referred to as different pathovars of X. campestris. Regardless of the origin or alternate hosts of strains of X. campestris pv. citrumelo, these strains are closely related based on DNA reassociation data presented here. In addition, few polymorphisms were observed when genomic DNA of strains of X. campestris pv. citrumelo were probed with the hrp gene cluster from X. campestris pv. vesicatoria. Since the hrp

genes are related to pathogenicity (Bonas, in press; Boucher et al., 1987; Lindgren et al., 1986), the lack of polymorphism would support pathogenicity similarities of the strains from citrus. The similarity values among these strains based on DNA reassociation differs from similarities generated by RFLP and justify the modification of the above hypothesis. Although several different pathovars of X. campestris may be represented among strains of X. campestris pv. citrumelo, these strains are all related. Thus, the weakly aggressive reactions caused by some strains of X. campestris pv. citrumelo are not the result of pathovars of X. campestris coincidentally associated with injury or lesions on citrus tissue.

Finally, it is possible that strains of X. campestris pv. citrumelo of all three aggressiveness types represent a single pathovar. The similarity of the hybridization pattern of restricted genomic DNA of strains of X. campestris pv. citrumelo probed with the hrp gene cluster supports this possibility. Pathovars of X. campestris are known to vary in their heterogeneity (Vauterin et al., 1990a). It is not unusual for a pathovar to display the amount of diversity observed here for X. campestris pv. citrumelo. For example, a recent study of X. campestris pv. begoniae included DNA reassociation data with a range of 85 to 100% for comparisons within the pathovar (Vauterin et al., 1990b). These values are similar to those presented

here for X. campestris pv. citrumelo. The diversity of pathogenic traits and genomic characteristics could be explained by a series of genomic recombination events. Experimentation designed to explore this possibility might explain some of the variation noted in these strains.

Another implication of the relatedness of strains of X. campestris pv. citrumelo concerns the use of RFLP analysis for bacterial characterization. Relative differences between strains, including differences between aggressiveness groups, were readily discerned using RFLP comparisons (Gabriel et al., 1989; Hartung and Civerolo, 1989). Moderately and weakly aggressive strains were especially diverse as compared by RFLP analysis (Graham et al., 1990c). Absolute differences between strains of X. campestris pv. citrumelo using DNA reassociation, however, were rather small and no differences were detected among aggressiveness types. An important conclusion from this comparison is that similarities derived by these two methods are not necessarily equivalent. These two techniques are best used in different contexts. Comparison by RFLP analysis is most useful for examination of relative differences between closely related strains and DNA reassociation for absolute differences between more distantly related strains.

The differences in similarity values obtained using RFLPs and DNA reassociation as well as other published techniques emphasize the importance of using a variety of

techniques to characterize bacteria (Vauterin et al., 1990a). While many techniques are applicable, only when several techniques have been used on a set of strains can a thorough understanding of the relationships among bacterial strains be achieved. Such an approach is essential when taxonomic revisions are proposed.

The use of RFLP analysis has been, and will continue to be, valuable in the characterization of bacterial strains. However, RFLP and DNA reassociation data are not necessarily equivalent and DNA reassociation is the currently accepted method of choice for species determination (Wayne et al., 1990). The comparison of bacterial strains by RFLP analysis is subject to bias from the number and choice of probes and the limited amount of the genome examined. For these reasons, RFLP comparisons cannot be substituted for DNA reassociation data at this time. The use of RFLP data in bacterial characterizations would be facilitated by the standardization of RFLP analyses. Research is needed to determine the importance of the number and choice of probes.

While DNA reassociation is best used for bacterial strains within the same species, restriction endonuclease analysis of infrequently occurring recognition sites is more useful to detect differences among closely related strains. Strains within groups A and B of X. campestris pv. citri were shown to have similar restriction endonuclease patterns, whereas each moderately and weakly aggressive

strain of X. campestris pv. citrumelo had a completely unique restriction pattern. In contrast to the genomic homogeneity of strains of X. campestris pv. citri groups A and B, both groups had diverse plasmid profiles. This example again illustrates the possible differences between techniques. Strains of X. campestris pv. citri group A were almost identical by restriction endonuclease analysis, yet the diversity of the plasmid profiles of these strains means that there are differences between these strains.

The origin and source of strains for outbreaks of Citrus bacterial spot remain unknown. The strains of X. campestris pv. citrumelo found in Florida have been shown here to be related by DNA reassociation, yet restriction endonuclease analysis indicates that no two strains are identical. It has been suggested here that strains of X. campestris pv. citrumelo are not opportunistic nor are they random representatives of pathovars merely associated with lesions on citrus leaves. The less aggressive strains of X. campestris pv. citrumelo may belong to a group of strains which cause relatively minor symptoms on a wide variety of hosts such as the ornamental strains investigated here. However, the aggressiveness of these strains on their noncitrus hosts is not clear. Further research into strains of X. campestris in Florida which cause symptoms on a wide variety of hosts may help explain the origin of Citrus bacterial spot. Perhaps an investigation into the nature of

the genetic variation in X. campestris pv. citrumelo will explain why, in a relatively short period of time, this group of strains were found to cause symptoms on citrus. In any case, more work must be done to explain the presence of this unusual group of bacteria.

APPENDIX

Table A-1. List of Xanthomonas campestris pathovars used in this study, their strain designation, and their source.

Pathovar	Strain designation		Source
	Lab. No.	DPI No.	
alfalfae	82-1		RES
campestris	33913^T		ATCC
citrumelo	F1	84-3048	ELC
citrumelo	F5	84-3162	DPI
citrumelo	F6	84-3401	DPI
citrumelo	F54	85-5436-1	DPI
citrumelo	F59	85-6572R-1-1	ELC
citrumelo	F86	85-8600-1	ELC
citrumelo	F94	85-6774-3	ELC
citrumelo	F100	85-12869	DPI
citrumelo	F228	84-169	DPI
citrumelo	F254	87-4665-1	DPI
citrumelo	F274	87-5789	DPI
citrumelo	F299	87-6200-2	DPI
citrumelo	F306	87-6314	ELC
citrumelo	F311	87-6345	DPI
citrumelo	F348	87-7222	DPI
citrumelo	F361	88-3851-3	DPI

Table A-1--continued.

Pathovar	Strain designation		Source
	Lab. No.	DPI No.	
citrumelo	F397	88-540	DPI
citrumelo		84-3166	DPI
citrumelo		85-6261	DPI
citrumelo		88-534	DPI
citrumelo		89-3274	DPI
citrumelo		90-4929-1	DPI
citrumelo		90-4929-2	DPI
citrumelo		90-4929-3	DPI
citrumelo		90-5429	DPI
citrumelo		90-5439-1	DPI
citrumelo		90-5532-1	DPI
citrumelo		90-5532-2	DPI
citrumelo		90-5560-1	DPI
citrumelo		90-5573-1	DPI
citri		86-3213	DWG
citri		86-3340	DPI
citri		86-9760-2	DPI
citri		86-9771	DPI
citri	T1		RES
citri	B64		ELC
citri	B69		ELC
citri	B80		ELC
citri	B84		ELC
citri	B93		ELC

Table A-1--continued.

Pathovar	Strain designation		Source
	Lab. No.	DPI No.	
citri	B148		ELC
fici	X151		ARC
maculifoliigardeniae	X22j		DPI
malvacearum	N		DWG
phaseoli	Xp 20[H]		ATCC
undetermined	X198		ARC
vesicatoria	Xv 56		RES
vesicatoria	Xv 75-3		RES

ARC=A. R. Chase, Department of Plant Pathology, University of Florida, Apopka, FL. ATCC= American type culture collection, Rockville, MD. DPI=Department of Plant Industry, Gainesville, FL. DWG=D. W. Gabriel, Plant Pathology, University of Florida, Gainesville, FL. ELC=E. L. Civerolo, U.S.D.A., Beltsville, MD. RES=R.E. Stall, University of Florida, Gainesville, FL.
[H]Holopathotype strain.
[T]Type strain of the species.

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BIOGRAPHICAL SKETCH

Dan Egel was born in Dayton, Ohio, on January 17, 1958, to Joan and William Egel. He attended Five Points Elementary and Junior High School and Park Hills High School where he graduated in 1976. In the summer of 1976 Dan was a foreign exchange student in São Leopoldo, Brazil. The following fall, Dan enrolled at Miami University, Oxford, Ohio, where he majored in botany and took courses in mycology and plant pathology from Dr. Martha Powell. Dan graduated with a B.S. from Miami University in 1980 and began a graduate program in forestry under Dr. Phil Pope at Purdue University in the fall of that year. At Purdue, Dan studied the interactions of a mycorrhizal fungus with the roots of green ash seedlings. After graduating from Purdue University in 1983 with a M.S., Dan took the position of laboratory manager of a soil testing laboratory in Terre Belle, CA. In September 1983, Dan enrolled in the graduate program at the University of California, Riverside, Plant Pathology Department. In Riverside, Dan worked with Dr. Robert Endo and Dr. Don Cooksey on the biological control of Cochliobolus sativus on Kentucky bluegrass and with Dr. John Menge on phytophthora root rot of citrus. In October 1985, Dan took a technical position with Dr. Jim Graham in Lake

Alfred, FL, where he continued to work with phytophthora root rot of citrus and mycorrhizae. In 1987, Dan enrolled in the graduate program at the University of Florida, Department of Plant Pathology, with Dr. Jim Graham and Dr. Robert Stall as coadvisors. Dan was married in 1987 to Denise Fardelmann and, in 1989, Denise gave birth to their son Sam.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert E. Stall, Chair
Professor of Plant Pathology

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Ann R. Chase
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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James H. Graham, Jr.
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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